Université de Sherbrooke

Prévention de la migration radio-induite des cellules cancéreuses du sein

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Thèse présentée à la Faculté de médecine et des sciences de la santé
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“Don't say you don't have enough time. You have exactly the same number of hours per day that were given to Helen Keller, Pasteur, Michaelangelo, Mother Teresa, Leonardo da Vinci, Thomas Jefferson, and Albert Einstein.”

— *H. Jackson Brown Jr.*

RÉSUMÉ

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Le cancer du sein triple négatif (TNBC) représente entre 15-20% des cancers du sein et est l'un des types les plus agressifs. De plus, un sous-groupe de ces patientes est résistant à la radiothérapie (RT) et développe fréquemment une récidive hâtive de la maladie. Des études précédentes ont démontré que l'inflammation induite par la RT accélère la progression du cancer et le développement des métastases. Cette hypothèse a donc été validée dans un modèle pré-clinique de TNBC en implantant les cellules de carcinome de souris triple négatives D2A1 dans les glandes mammaires de la souris Balb/c. Premièrement, la tumeur primaire à été irradiée à une dose sous-curative une semaine post-implantation des cellules. En deuxième lieu, le tissu mammaire de la souris a été pré-irradié avant d'implanter les cellules cancéreuses afin de bien discerner l'effet du microenvironnement irradié sur celles-ci. Ces deux modèles ont mené à une augmentation significative des cellules tumorales circulantes ainsi que du nombre de métastases pulmonaires. Plusieurs molécules inflammatoires dont l'interleukine-1 bêta (IL-1β), l'interleukine-6 (IL-6) ou encore la cyclooxygénase 2 (COX-2) ont été identifiées comme facteurs clés impliqués dans la migration radio-induite des cellules cancéreuses du sein. Conséquemment, un inhibiteur large-spectre comme la chloroquine (CQ), entre autres utilisé comme traitement anti-malarien et anti-inflammatoire, a su prévenir ces effets secondaires associés à la RT. Étant donné que l'action de la CQ est peu sélective, une répression de l'expression de l'ARNm de la métalloprotéinase (MMP) de membrane de type 1 (MT1-MMP), une MMP de surface impliquée notamment dans la migration cellulaire, l'invasion tumorale et l'angiogenèse, a été réalisée afin d'éclaircir le mécanisme d'inhibition des métastases radio-induites. Cette répression de la MT1-MMP prévient la formation des métastases pulmonaires radio-induites, démontrant ainsi un des mécanisme important de l'invasion radio-induite. Ce résultat confirme donc l'importance de la MT1-MMP dans ce phénomène et son potentiel comme biomarqueur de prédiction de l'efficacité des traitements de RT, particulièrement chez les patientes atteintes de TNBC.

Mots clés : Cancer du sein triple négatif (TNBC), radiation, invasion, migration, inflammation, métalloprotéinase membranaire de type 1 (MT1-MMP), métastases

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15-PGDH	15-hydroxyprostaglandine déshydrogénase / *15-hydroxyprostaglandin dehydrogenase*
α-SMA	actine du muscle lisse alpha / *smooth muscle actin alpha*
ADN	acide désoxyribonucléique / *deoxyribonucleic acid*
ANOVA	analyse de variance / *analysis of variance*
ATCC	*American Type Culture Company*
BED	dose biologique équivalente / *biologically effective dose*
BLBC	cancer du sein du type basal / *basal-like breast cancer*
BSA	albumine sérique bovine / *bovine serum albumin*
CD31	*cluster of differentiation 31*
Cdt1	*chromatin licensing and DNA replication factor 1*
Co	cobalt / *cobalt*
COX-1	cyclooxygéase-1 / *cyclooxygenase-1*
COX-2	cyclooxygéase-2 / *cyclooxygenase-2*
cPLA2	phospholipase A2 cytosolique / *cytosolic phospholipase A2*
CQ	chloroquine / *chloroquine*
CTC	cellule tumorale circulante / *circulating tumor cell*
CTL	contrôle / *control*
DMEM	*Dulbecco modified Eagle's medium*
ECL	*enhanced chemiluminescence*
ECM	matrice extracellulaire / *extracellular matrix*
EMT	transition épithélio-mésenchymateuse / *epithelial-mesenchymal transition*
ER	récepteur de l'œstrogène / *estrogen receptor*
FACS	*fluorescence-activated cell sorting*
Fas-L	ligand Fas / *Fas ligand*
FBS	sérum foetal bovin / *foetal bovine serum*
FGF	facteur de croissance fibroblastique / *fibroblast growth factor*

FIH-1α	facteur inhibiteur du HIF-1α / *factor inhibiting HIF-1α*
FITC	*fluorescein isothiocyanate*
FRQ-S	Fonds de Recherche du Québec - Santé
FRSQ	Fonds de Recherche en Santé du Québec
FUCCI	*fluorescent ubiquitinated-based cell cycle indicator*
GAPDH	glycéraldéhyde-3-phosphate déshydrogénase / *glyceraldehyde-3-phosphate dehydrogenase*
GBM	glioblastome multiforme / *glioblastoma multiforme*
Gy	grey / *grey*
H&E	hématoxyline et éosine */ haematoxylin and eosin*
HER2	récepteur pour les facteurs de croissance épidermiques humains / *human epidermal growth factor receptor 2*
HIF-1	facteur induit par l'hypoxie 1 / *hypoxic-inducible factor 1*
HPRT	hypoxanthine-guanine phosphoribosyltransférase / *hypoxanthine-guanine phosphoribosyltransferase*
HRP	peroxydase de raifort */ horse radish peroxidase*
ICAM-1	molécule d'adhérence intercellulaire *1 / intercellular adhesion molecule 1*
ICE	enzyme de conversion de l'interleukine-1 bêta / *interleukin-1 beta converting enzyme*
ID	numéro d'identification / *identification number*
IHC	immunohistochimie / *immunohistochemistry*
IL	interleukine / *interleukin*
i.p.	intrapéritonéale / *intraperitoneal*
IRR	irradié / *irradiated*
ITCN	*Image-based Tool for Counting Nuclei*
LC3	*microtubule-associated protein 1 light chain 3*
LC-MS/MS	chromatographie en phase liquide couplée à la spectrométrie de masse / *liquid chromatography-mass spectrometry*
LSD	*least significant difference*
mAG	*monomeric Azami green*

MCP-1	protéine chimioattractive des monocytes de type 1 / *monocyte chemotactic protein 1*
MEM	*modified Eagle's medium*
MIP-2	protéine inflammatoire des macrophages de type 2 / *Macrophage inflammatory protein 2*
mKO2	*monomeric Kusabira orange 2*
MMP	métalloprotéinase de matrice / *matrix metalloproteinase*
mRNA	acide ribonucléique messager / *messenger ribonucleic acid*
NaCl	chlorure de sodium / *sodium chloride*
NSAID	Anti-inflammatoire non-stéroïdien / *non-steroidal anti-inflammatory drug*
OCT	*optimum cutting temperature*
PAVI	plateforme d'analyse et de visualisation d'image
PBS	tampon phosphate salin / *phosphate buffered saline*
PFA	parafolmaldéhyde / *paraformaldehyde*
PGD2	prostaglandine D2 / *prostaglandin D2*
PGE2	prostaglandine E2 / *prostaglandin E2*
PLA2	phospholipase A2 / *phospholipase A2*
PVDF	fluorure de vinylidène / *polyvinylidene difluoride*
plp	lipophiline / *lipophilin*
PR	récepteur de la progestérone / *progesterone receptor*
qPCR	amplification en chaîne par polymérase quantitative en temps réel / *quantitative real-time polymerase chain reaction*
ROS	espèces réactives de l'oxygène / *reactive oxygen species*
RT	radiothérapie / *radiotherapy*
SCID	déficit immunitaire combiné sévère / *severe combined immunodeficiency*
SEM	erreur type / *standard error of the mean*
shRNA	petits acides ribonucléiques en épingle à cheveux / *small hairpin ribonucleic acid*
TAM	*macrophages associés aux tumeurs / tumor-associated macrophages*

TBE	*tumor-bed effect*
TGF-β	facteur de croissance transformant-1 bêta */ transforming growth factor beta 1*
TNBC	cancer du sein triple négatif / *triple negative breast cancer*
TNF-α	facteur de nécrose tumorale alpha / *tumor necrosis factor alpha*
TRITC	*tetramethylrhodamine isothiocyanate*
UBC	Ubiquitine C / *ubiquitin C*
uPAR	récepteur de l'urokinase / *urokinase receptor*
VCAM-1	molécule d'adhérence aux cellules vasculaires 1 */ vascular cell adhesion molecule 1*
VEGFA	*facteur de croissance endothéliale vasculaire de type A* / vascular endothelial growth factor A
VH	véhicule / *vehicle*
VLA-4	antigène très tardif de type 4 */ very late antigen-4*
VSVG	virus de la stomatite vésiculaire / *vesicular stomatitis Indiana virus*

INTRODUCTION

1.1 Le cancer du sein

Au Canada, le cancer du sein est la forme de cancer la plus répandue chez la femme. Selon des statistiques établies en 2010, une Canadienne sur neuf sera atteinte d'un cancer du sein dans sa vie et une sur 30 en mourra (Société canadienne du cancer, 2016a). Les hommes peuvent aussi en être atteints, mais il s'agit de moins de 1% des cas (Société canadienne du cancer, 2016b). Bien que l'avènement de la mammographie ait significativement diminué la mortalité associée au cancer du sein dans les pays développés grâce à sa détection plus précoce, l'incidence de ce type de cancer continue tout de même d'augmenter.

Le cancer du sein se manifeste sous différentes formes, affectant différentes structures du sein. Dans la majorité des cas, celui-ci se développe dans les tissus épithéliaux mammaires comme les canaux galactophores (carcinome canalaire) ou encore dans les lobules (carcinome lobulaire). Dépendant de la nature infiltrante du cancer, il peut être *in situ* (confiné dans le tissu d'origine) ou infiltrant (propagé dans les tissus voisins) (Société canadienne du cancer, 2016c).

Le cancer du sein, comme tout autre cancer, est associé à deux catégories de facteurs de risques, soit les prédispositions génétiques ou encore les facteurs environnementaux. Parmi les facteurs génétiques, les antécédents personnels ou familiaux de cancer du sein sont les facteurs de risques les plus élevés. Les femmes ayant une mutation du gène *BRCA* ont un risque pouvant aller jusqu'à 80 % d'être un jour atteinte d'un cancer du sein (Société canadienne du cancer, 2016d). Plus précisément, une mutation du gène *BRCA* entraînera une recombinaison homologue de l'ADN inefficace causant une accumulation d'aberrations génétiques pouvant mener jusqu'à une prolifération cellulaire incontrôlée et ultimement jusqu'au cancer (Langlands *et al.*, 2013). En ce qui concerne les facteurs externes, l'obésité, l'alcool, le tabagisme et l'exposition aux rayonnements ionisants sont des exemples de facteurs environnementaux qui peuvent aussi augmenter le risque de développer un cancer du sein (Société canadienne du cancer, 2016d).

De par son hétérogénéité, le cancer du sein est une maladie complexe. Des

paramètres cliniques standards comme la taille de la tumeur, le nombre de ganglions touchés, l'âge de la patiente, le nombre de métastases ou encore la présence de récepteurs hormonaux comme les récepteurs de l'estrogène (ER), de la progestérone (PR) ou encore le récepteur du facteur de croissance épidermique humain (HER2) sont caractérisés chez chaque patiente. La structure et la forme des cellules, le pléomorphisme nucléaire (forme atypique des noyaux) ainsi que la fréquence des mitoses sont aussi évalués histologiquement pour établir le grade de la tumeur ainsi qu' un pronostic. Les tumeurs de haut grade sont généralement peu différenciées et ont tendance à se propager rapidement (Société canadienne du cancer, 2016e). Les sites métastatiques préférentiels associés au cancer du sein sont les os (48%), le foie (27%), le cerveau (17%) et les poumons (23%) (Soni *et al.*, 2015).

Plusieurs études ont mis au jour l'hétérogénéité moléculaire du cancer du sein en identifiant différents paramètres clinicopathologiques. Ceux-ci sont modulés en fonction de certains sous-groupes de gènes exprimés (Perou *et al.*, 2000; Eroles *et al.*, 2012). La signature moléculaire s'est avérée avoir un impact direct sur la réponse au traitement ainsi que la survie du patient (Gluz *et al.*, 2009; Eroles *et al.*, 2012). Une nouvelle classification en 6 sous-groupes a émergée de ces études, soit le cancer du sein de type basal (*basal-like)*, surexprimant le HER2 (*HER2-enriched*), apparenté aux tissus normaux (*normal breast-like*), luminal A (*luminal A*), luminal B (*luminal B*) ou encore ayant une faible expression de claudine (*claudin-low*) (Eroles *et al.*, 2012). Les principales caractéristiques de ces sous-groupes sont résumées dans le Tableau 1.

Tableau 1. Classification simplifiée des sous-types de cancer du sein (adapté de Eroles et al., 2012).

Sous-type moléculaire	Fréquence	Statut hormonal	Grade	Pronostic
Luminal A	50-60%	ER+, PR+, HER2-	Bas	Excellent
Normal breast-like	5-10%	ER-/+, HER2-	Bas	Intermédiaire
Luminal B	10-20%	ER-/+, PR-/+, HER2-/+	Intermédiaire/haut	Intermédiaire/Mauvais
HER2-enriched	10-15%	ER-, PR-, HER2+	Haut	Mauvais
Basal-like	10-20%	ER-, PR- HER2-	Haut	Mauvais
Claudin-low	12-14%	ER-, PR-, HER2-	Haut	Mauvais

Différentes thérapies sont utilisées de routine pour le traitement du cancer du sein. La chirurgie est le traitement de choix et est indiquée dans presque tous les cas en absence de métastases à distance. Dans certaines circonstances, la femme peut elle-même choisir le type de chirurgie qu'elle subira, soit une mastectomie partielle, une mastectomie totale avec biopsie du ganglion sentinelle ou encore une mastectomie radicale modifiée (mastectomie totale et dissection axillaire). Un traitement de chimiothérapie sera administré à la patiente subséquemment à la chirurgie de la tumeur dans les cas de risque de récidive élevée, de cancer avancé ou rechute et de plus en plus, de la chimiothérapie pré-opératoire est administrée chez les patientes avec métastases ganglionnaires. Le traitement de chimiothérapie standard de première ligne utilisé au Centre hospitalier universitaire de Sherbooke pour le cancer du sein est normalement une combinaison de cyclophosphamide, épirubicine et 5-fluorouracil. Dépendamment des récepteurs exprimés dans chaque cas de cancer du sein, une hormonothérapie peut être administrée ou encore une thérapie avec des anticorps monoclonaux ciblant le HER2 (Société canadienne du cancer, 2016f). Les biphosphonates sont aussi largement utilisés dans les cas de métastases osseuses découlant du cancer du sein, ceux-ci réduisant le risque de fractures et de douleurs osseuses, augmentant donc la survie et la qualité de vie des patientes (EBCTCG, 2015). La radiothérapie est aussi un traitement très important dans le cancer du sein. Celle-ci sera discutée plus en détails à la section 1.2.

1.1.1 Le cancer du sein triple négatif

Le cancer du sein triple négatif (TNBC) représente entre 10-20% des cancers du sein. Son appellation triple négatif fait référence à l'absence ou la très faible expression des ER, PR et HER2. Ce type de cancer du sein est couramment diagnostiqué chez les femmes plus jeunes et à une prévalence plus élevée chez les Afro-Américaines (Moran, 2015). Une revue de littérature sur le TNBC réalisée par Gluz *et al*. résume les caractéristiques principales de ce type de cancer du sein. Brièvement, plus de 90% des TNBC sont de phénotype infiltrant, de haut grade histologique, présentent un index mitotique élevé ainsi qu'une zone nécrotique centrale (Gluz *et al.*, 2009). De plus, une différence d'expression a été démontrée pour plusieurs gènes impliqués entre autres dans la prolifération, l'activité des kinases, la

réparation de l'ADN et l'apoptose chez les TNBC en comparaison avec les cancers du sein exprimant les ER et HER2 (Gluz *et al.*, 2009). Les patientes ayant des cancers ER ou HER2-positifs peuvent bénéficier d'une thérapie ciblée comme de l'hormonothérapie ou encore des anticorps monoclonaux ciblant spécifiquement ces récepteurs (Société canadienne du cancer, 2016f). Malheureusement, le TNBC est généralement associé avec un mauvais pronostic de survie, ou risque de décès plus élevé, en raison de son phénotype agressif menant souvent à un développement rapide de métastases ainsi que par l'impossibilité d'utiliser des thérapies ciblées.

Le TNBC est souvent appelé cancer de type basal (BLBC) à tort, car il existe une discordance entre ces deux groupes. Les BLBC sont dans la majorité des cas triples négatifs, mais sont aussi caractérisés par une augmentation des cytokératines de type 5, 6 et 17 (Gluz *et al.*, 2009; Eroles *et al.*, 2012). Par contre, certains BLBC expriment tout de même les récepteurs ER ou HER2 de même que plusieurs TNBC ne surexpriment pas les cytokératines typiques associées aux BLBC. Bien qu'il n'y ait aucune évidence moléculaire établie que les TNBC soient divisés en deux groupes distincts, il est connu que certains d'entre eux répondent positivement aux traitements alors que d'autres non. Par conséquent, deux catégories générales de TNBC en découlent, soit les bons et les mauvais répondants aux traitements.

Plusieurs études ont démontrées que les TNBC ont une prédilection à se disséminer dans les organes viscéraux et le cerveau alors que les cancers du sein positifs pour les récepteurs hormonaux vont plutôt disséminer vers les os (Kennecke et al., 2010). Le moment de récidive le plus fréquent chez les TNBC se situe pendant les trois premières années post-traitement. En ce qui concerne les décès associés, ceux-ci se produisent majoritairement dans les 5 premières années suivant le diagnostic initial. Cependant, aucune différence significative entre les TNBC et les cancers récepteurs hormonaux-positifs n'est observée pour la survie globale après avoir franchi le cap de 10 ans de suivi sans récidive (Gluz *et al.*, 2009). Afin de diminuer la récidive précoce et d'identifier de nouvelles cibles thérapeutiques, une meilleure compréhension de la biologie du TNBC et la découverte de biomarqueurs sont impératifs (Gluz *et al.*, 2009; Mahamodhossen *et al.*, 2013).

1.2 La radiothérapie

La radiothérapie (RT) est utilisée chez un très grand nombre de patientes atteintes de cancer du sein car elle augmente significativement la survie de celles-ci (EBCTCG, 2005). La RT est administrée suivant la très vaste majorité des cas de mastectomie partielle du sein (thérapie adjuvante), mais peut aussi être recommandée après une mastectomie radicale modifiée ou totale, dépendamment entre autres de l'extension locale et du nombre de ganglions touchés (Recht *et al.*, 2001). Bien que cela soit plus rare dans le traitement du cancer du sein, la RT peut parfois être administrée avant la chirurgie (thérapie néoadjuvante) afin de réduire le volume tumoral initial et ainsi faciliter la chirurgie qui s'en suivra (Palta *et al.*, 2012; American Cancer Society, 2014).

La RT consiste à l'utilisation de rayons ionisants qui induisent des dommages à l'ADN des cellules et les détruisent. Son but premier est d'éliminer les cellules cancéreuses résiduelles nichées dans le site de résection de la tumeur qui sont normalement non-détectables en imagerie. Les radiations ionisantes ciblent principalement les cellules à division rapide, donc affectent préférentiellement les cellules cancéreuses qui ont un métabolisme plus rapide que les cellules saines (American Cancer Society, 2014).

Deux types principaux de rayons ionisants sont utilisés dans le traitement du cancer, soit les photons, incluant les rayons-X et les rayons gamma ou encore les radiations particulaires, incluant les électrons, les protons, les neutrons, les hadrons ou encore les particules alpha et bêta. Plus le spectre d'énergie est élevé, plus la radiation pénètre en profondeur dans les tissus. De ce fait, le type de radiation peut être choisi en fonction du type de cancer mais celui-ci est surtout choisi de manière à obtenir la meilleure dosimétrie, c'est-à-dire obtenir la dose optimale au volume ciblé tout en épargnant au maximum les tissus sains.

Le traitement de RT est normalement administré pendant 5 à 8 semaines à raison de 5 jours par semaines parce que les petites fractions répétées sont moins dommageables pour les tissus sains qu'une seule fraction d'une dose totale équivalente. Ce principe est basé sur la capacité intrinsèque de la cellule à réparer les dommages à l'ADN. Les standards de fractionnement de la dose peuvent être différents selon les institutions. Le traitement de RT de base du cancer du sein après une chirurgie conservatrice au Centre hospitalier

universitaire de Sherbrooke est de 42,56 Gy en 16 fractions de 2,66 Gy qui peut varier un peu dépendant de la taille du sein tandis que le standard américain est plutôt de 50 Gy en 25 fractions de 2 Gy. Un complément d'irradiation ou «*boost*» au lit tumoral peut aussi être recommandé dans certains cas. Étonnamment, même après de nombreuses études prouvant les bénéfices de la RT (EBCTCG et al., 2005), plusieurs femmes décident de ne pas en bénéficier en raison de problèmes d'accessibilité des installations pendant de trop longues périodes occasionnant des absences au travail répétées. En lien, la possibilité de raccourcir les traitements de RT en augmentant les doses par fraction et ainsi optimiser le rapport coût-efficacité est actuellement sous investigation. Plusieurs de ces études ont d'ailleurs démontré un contrôle locorégional à long terme, des toxicités et des effets cosmétiques résultants équivalents au régime standard. Par conséquent, les protocoles d'hypofractionnement sont de plus en plus encouragés dans la pratique clinique (Brown *et al.*, 2015).

La RT débute généralement quelques semaines après la chirurgie suivant la guérison de la plaie. Toute la glande mammaire est irradiée, incluant les aires ganglionnaires et la paroi thoracique au besoin. Le complément d'irradiation dans le lit tumoral est recommandé chez les femmes ayant des marges focalement positives après le retrait de la tumeur, une tumeur de haut grade, de plus de 5 cm de diamètre ou trop près de la paroi thoracique (Société canadienne du cancer, 2016g). En ce qui concerne la RT suite à une mastectomie, le site post-chirurgie est irradié seulement si le cancer affecte 4 ganglions lymphatiques ou plus, si la tumeur mesure plus de 5 cm de diamètre ou si celle-ci a envahi la paroi thoracique ou la peau (Recht *et al.*, 2001; Société canadienne du cancer, 2016g).

Il est important de mentionner que les doses de RT ne sont pas calculées pour éliminer toutes les cellules cancéreuses, mais plutôt pour optimiser l'efficacité du traitement en minimisant les dommages infligés aux tissus normaux. L'optimisation de la RT permettrait notamment d'infliger une dose plus élevée aux cellules cancéreuses en limitant les effets secondaires sur les tissus normaux. Les effets secondaires de la RT se manifestent soit en phase aiguë ou en phase tardive. Les effets aigus se produisent pendant le traitement et se poursuivent jusqu'à 2 à 3 semaines post-irradiation. Les tissus sains à division rapide comme les muqueuses sont particulièrement touchés pendant cette phase (Booth et al.,

2012). La phase aiguë se traduit majoritairement par une réponse inflammatoire pouvant causer de l'érythème, des mucosites (inflammation des muqueuses), des nausées et des diarrhées (Yang *et al.*, 2011). Cette inflammation radio-induite est considérée comme un facteur limitant de la dose (Yang *et al.*, 2011). Par exemple, la majorité des patientes développent une dermatite, qui se manifeste par des rougeurs et un œdème cutané. Une desquamation humide peut être observée dans les cas les plus graves. Une fatigue est aussi un effet secondaire commun associé à la RT (Siva *et al.*, 2015). Également, le lymphoedème du bras, soit une accumulation de liquide lymphatique, survient chez environ 10 à 50% des patientes traitées (Siegel *et al.*, 2012). Cette complication tend à empirer avec le nombre de ganglions retirés (Coles *et al.*, 2005). Cette thèse se concentre principalement sur la réponse inflammatoire radio-induite aiguë. Ces effets néfastes potentiels sur la progression du cancer seront discutés plus en détails dans la section 1.2.2 portant sur l'invasion radio-induite.

La RT peut aussi occasionner de la toxicité chronique qui peut entraîner des complications beaucoup plus sérieuses se traduisant normalement par une atteinte ou une perte de fonction du tissu touché. Les effets pulmonaires associés aux radiations comme la fibrose ou encore la pneumonite radique, une inflammation pulmonaire, et les risques de complications cardiaques sont fréquents. Ces effets secondaires sont directement attribués au volume de l'organe irradié. En lien, plusieurs études ont démontré qu'il y a beaucoup plus de complications cardiaques chez les femmes ayant une tumeur du sein gauche, en raison de la difficulté d'éviter le cœur dans ces cas précis (Recht *et al.*, 2001). De plus, une méta-analyse démontre que même si les risques de décès associés au cancer diminuent avec la RT post-chirurgie, ceux-ci peuvent être annulés par les décès associés à des complications cardiaques, résultant en une survie globale équivalente au final (Cuzick *et al.*, 1987). Il est à prendre en considération que ces données proviennent de patientes traitées avec des équipements moins précis qu'aujourd'hui. Avec les nouvelles technologies de modélisation des plans de traitements et des accélérateurs, la toxicité reliée à ces organes est beaucoup mieux contrôlée et ne s'avère plus vraiment un problème.

Bien que le risque soit assez faible dans les cas de RT du sein, il est connu que la radiation peut aussi entraîner des cancers secondaires (Brown *et al.*, 2015). Par exemple,

lorsque la cellule ne peut réparer un dommage important, la cellule-fille sera porteuse du même dommage. Ce dommage sera ainsi transmis chez tous les clones et peut entraîner une néoplasie radio-induite. Les cancers du sein radio-induits se manifestent très rarement, et de façon tardive, soit plus de 10 ans post-irradiation (Yi *et al.*, 2009). Par contre, lorsque traité à l'âge enfant ou jeune adulte avec de la RT, ce risque augmente significativement, car le tissu mammaire est toujours en maturation, donc plus sensible aux radiations ionisantes (Castiglioni *et al.*, 2007).

1.2.1 L'inflammation radio-induite

L'inflammation radio-induite est un effet secondaire qui apparaît à différents degrés chez toutes les patientes pendant le traitement de RT (Yi *et al.*, 2009; Gallet *et al.*, 2011). Bien que cet effet secondaire se résorbe normalement après quelques semaines (Yi *et al.*, 2009), l'inflammation radio-induite peut entraîner d'autres complications qui pourraient diminuer les chances de survie de la patiente à long terme.

Au niveau moléculaire, l'inflammation se traduit par une libération de cytokines menant aussi à la création d'espèces réactives de l'oxygène (ROS) pouvant causer des dommages à l'ADN. Les cytokines sont des polypeptides solubles sécrétés par plusieurs types de cellules agissant sur elles-mêmes (autocrine), sur les tissus avoisinants (paracrine) ou encore sur des tissus distants (endocrine) (Barcellos-hoff, 1998). La RT a une action anti-inflammatoire et immunosuppressive de par la destruction de cellules effectrices, mais cette destruction entraîne aussi une réaction inflammatoire et la libération de chimiokines (cytokines chimiotactiques) qui provoquent un effet opposé. Une revue à ce sujet à été réalisée par McBride *et al.* (McBride et al., 2004).

Parmi les cytokines libérées post-RT, le TNF-alpha (TNF-α) a été identifié comme molécule pro-inflammatoire clé au niveau de l'inflammation radio-induite (Hill *et al.*, 2011). Plusieurs autres cytokines comme l'interleukine-1bêta (IL-1β), l'interleukine-6 (IL-6), la molécule d'adhérence intercellulaire-1 (ICAM-1), la molécule d'adhérence aux cellules vasculaires-1 (VCAM-1), la E-sélectine, la protéine chimioattractive des

monocytes de type 1 (MCP-1), le facteur de croissance transformant-1 bêta (TGF-β), le ligand Fas (Fas-L), le facteur de croissance fibroblastique (FGF) ou encore les métalloprotéinases-2 et 9 (MMP-2, -9) sont très bien reconnus pour être augmentés post-irradiation (Lee *et al.*, 2011; Yang *et al.*, 2011; Schaue *et al.*, 2012; Mukherjee *et al.*, 2014). Bien qu'une panoplie de cytokines radio-induites ait déjà été identifiée, leur action précise sur les cellules cancéreuses ou encore au sein du microenvironnement tumoral irradié reste peu connue.

Plusieurs cytokines inflammatoires libérées post-irradiation ont été documentées pour leur effet favorisant la migration et l'invasion des cellules cancéreuses, promouvant ainsi la progression tumorale (Desmarais et al., 2012; Bouchard et al., 2013). L'inflammation radio-induite peut aussi entraîner l'activation des macrophages tissulaires. Lorsque les macrophages sont activés, les cytokines qu'ils libèrent peuvent engendrer des lésions génétiques dans les cellules, et ce de plusieurs heures à plusieurs semaines post-irradiation. En plus de promouvoir l'invasion, l'inflammation radio-induite peut aussi mener à une instabilité chromosomique (Mukherjee *et al.*, 2014).

Outre la libération de cytokines, plusieurs études démontrent que l'expression de la cyclooxygénase-2 (COX-2) est significativement augmentée en cas d'inflammation, de cancer ou post-irradiation (Khan *et al.*, 2007; Yang *et al.*, 2011; Desmarais *et al.*, 2015). Les dérivés de cet enzyme, les prostaglandines, particulièrement la prostaglandine E2 (PGE2), sont connues comme étant responsables des symptômes de la douleur, de fièvre et de l'œdème typiques d'une réponse inflammatoire. La COX-2 est aussi bien connue pour son implication dans la progression du cancer et le développement des métastases (Greenhough *et al.*, 2009). Une étude réalisée par Jaal *et al.* à mis en évidence l'induction de la COX-2 en phase aiguë post-RT dans un modèle d'irradiation de la vessie de souris (Jaal and Dörr, 2006). Cette étude supporte l'implication potentielle de cette enzyme dans les cas de complications post-RT spécifiquement associées à cette même phase (Jaal and Dörr, 2006).

En bref, l'inflammation radio-induite promeut l'activation de macrophages, la libération de cytokines et l'induction de la COX-2. Ces éléments sont associés à la promotion de l'invasion cellulaire, le développement de métastases et l'instabilité

génomique. Il est évident que la prévention de l'inflammation radio-induite pourrait faire une différence dans le traitement du cancer et réduire les risques de récidive.

1.2.2 L'invasion radio-induite

Le lien biologique entre l'exposition aux radiations et les dommages à l'ADN a été établi il y a plusieurs décennies. Depuis, plusieurs nouvelles évidences ont complètement révolutionné cette théorie. Les effets néfastes des radiations ne se limitent pas seulement à l'initiation potentielle de nouvelles néoplasies, mais peuvent aussi contribuer à la croissance et à la progression tumorale d'un cancer déjà existant, voire promouvoir le développement de métastases (Barcellos-Hoff, 2013; Mukherjee *et al.*, 2014). Il est toutefois très difficile d'établir une conclusion ferme sur le phénomène étant donné que les données cliniques sont très hétérogènes (âge, ethnie, sexe, sous-type de tumeur, stade, grade, nombre de métastases, dose de RT, nombre de fractions, zone irradiée, type de rayonnement, chimiothérapie concomitante, chirurgie etc.), ce qui rend la comparaison des études difficile. Il est aussi très important de mentionner que la tumeur est un écosystème dynamique composé certes de cellules tumorales, mais aussi étroitement régulé avec les cellules du microenvironnement à savoir les cellules endothéliales, les cellules immunitaires ou encore les fibroblastes. L'étude de l'invasion radio-induite se doit donc d'établir les faits autant du point de vue des cellules cancéreuses que du microenvironnement irradié. Bien sûr, les deux composantes sont étroitement liées et s'influencent mutuellement.

1.2.2.1 L'invasion radio-induite par les cellules cancéreuses

L'hypothèse que la radiation puisse augmenter le potentiel métastatique des cellules cancéreuses a fait l'objet de plusieurs revues de littérature (Rofstad *et al.*, 2004; Moncharmont *et al.*, 2014; Fujita *et al.*, 2015). Afin d'étudier la capacité des photons à augmenter l'invasion, un bon nombre de lignées cellulaires dérivées de cancers humains ont été testées utilisant différents modèles *in vitro* et *in vivo*. La revue réalisée par le Fujita *et* al. met en évidence l'augmentation significative des propriété de migration et/ou d'invasion

radio-induites dans plusieurs lignées cellulaires humaines incluant le sein, le colon, le cerveau, la prostate, le foie ou encore le pancréas, pour ne nommer que ceux-là (Fujita *et al.*, 2015).

La cause de l'augmentation de l'invasion radio-induite ayant été le plus étudié est sans aucun doute l'induction de mutations génétiques (Moncharmont *et al.*, 2014; Fujita *et al.*, 2015). Le processus d'invasion cellulaire est divisé en deux types de motilité distinctes soit mésenchymale et amoeboïde, aussi appelées modalités protéase-dépendante et protéase-indépendante (Sahai, 2005; Fujita et al., 2015). Ces phénomènes sont rarement exclusifs chez la cellule et arrivent souvent les deux en même temps ou en alternance. C'est pour cette raison qu'il est difficile de freiner l'invasion cellulaire en ciblant les protéines impliquées dans l'une ou l'autre des deux modalités seulement. Certains gènes sont connus pour être fortement liés avec l'invasion protéase-dépendante (mésenchymale). Par exemple, les petites protéines G Rac1 et Ras de la famille des Rho GTPases sont connues pour réguler plusieurs de ces gènes. Une mutation du gène *RAS* induisant son expression est reconnue pour augmenter l'oncogenèse, car Ras est un messager essentiel dans plusieurs voies de signalisation impliquées entre autres dans la prolifération, la survie, la différenciation mais aussi la migration et l'invasion cellulaire. La dérégulation de cette voie de signalisation post-irradiation a entre autres été étudiée dans un modèle de souris utilisant les cellules humaines Caski de cancer du col de l'utérus (Su et al., 2012). Dans le même ordre d'idées, une mutation du gène *PTEN* peut aussi affecter l'agressivité des cellules cancéreuses. *PTEN* est un gène suppresseur de tumeur qui est souvent muté et délété dans plusieurs cas de cancer. La protéine encodée par ce gène est reconnue pour augmenter l'activité des Rho GTPases ainsi que l'expression de certaines protéases, augmentant donc l'invasion des cellules cancéreuses. L'implication de *PTEN* dans le phénomène d'invasion radio-induite a notamment été démontrée dans un modèle de sphéroïdes avec des cellules tumorales humaines de glioblastome ainsi que dans un modèle de souris immunodéficiente impliquant des cellules thyroïdiennes (Park et al., 2006; Burrows et al., 2013).

Une autre hypothèse expliquant potentiellement le phénomène d'invasion radio-induite des cellules cancéreuses est le manque d'oxygène au niveau de la tumeur post-RT. Les ROS créés par la radiolyse de l'eau réagissent avec l'oxygène, font diminuer celle-ci et

produisent un environnement hypoxique. L'hypoxie ainsi causée par l'irradiation augmente la stabilisation du facteur induit par l'hypoxie de type 1 (HIF-1) au noyau des cellules cancéreuses. Le facteur de transcription HIF-1 induit ensuite l'expression de gènes favorisant la tumorigenèse, l'angiogenèse, l'anti-apoptose, la glycolyse, les métastases, la libération de cytokines ou encore de facteurs de croissance (Palazon et al., 2014). À leur tour, ces facteurs recrutent des cellules immunitaires qui favorisent aussi l'invasion des cellules cancéreuses (Gu et al., 2015). La surexpression du HIF-1 est observée au niveau de plusieurs types de cancer comme le sein, le poumon, le cerveau ou encore la prostate, et est corrélée avec un mauvais pronostic de survie (Jia *et al.*, 2011; Palazon *et al.*, 2014). De plus, son expression est associée à une résistance à la RT, de par la production de ROS post-irradiation résultante qui est en grande partie responsable des dommages induits à l'ADN (Moon *et al.*, 2007).

Une étude a démontré qu'une diminution de l'expression du gène *HIF1A* chez des modèles expérimentaux de plusieurs types de cancer réduit la vascularisation et la masse tumorale, augmente l'apoptose, et cause un retard de l'apparition des métastases (Du *et al.*, 2008). Plusieurs études associent aussi HIF-1 comme facteur de mauvais pronostic par sa capacité à induire la transition épithélio-mésenchymateuse (EMT) chez les cellules cancéreuses, promouvant ainsi l'invasion et le développement de métastases. La EMT est le passage de la cellule épithéliale vers un phénotype fibroblastique caractérisé entre autres par une perte d'adhésion et une diminution de l'expression de la E-cadhérine. L'acquisition de ces caractéristiques facilitent la dispersion des cellules cancéreuses (Frixen et al., 1991). Plusieurs groupes ont démontré que la EMT est un mécanisme de résistance à la RT dans plusieurs cancers, incluant le cancer du sein (Zhang *et al.*, 2011; Zhou *et al.*, 2011). L'étude de Zhou *et al.* démontre qu'une dose de 2 Gy induit la EMT chez des cellules humaines d'adénocarcinome du sein, colorectal et pulmonaire. Il est intéressant de mentionner que 2 lignées cellulaires différentes pour chaque type de cancer ont été testées, soit une à faible potentiel métastatique et une à haut potentiel métastatique. De manière générale, cette étude démontre que la EMT radio-induite est plus facile à induire chez les cellules à haut potentiel métastatique (Zhou *et al.*, 2011).

Outre la EMT, la RT peut aussi induire un phénotype de type migratoire chez la cellule cancéreuse (Madani *et al.*, 2008). Ce phénomène où la cellule se divise ou migre de manière exclusive, se nomme dichotomie de prolifération/migration ou «*Go or Grow*» (Giese *et al.*, 1996; Hatzikirou *et al.*, 2012). Cette évolution phénotypique rapide exclue l'hypothèse de mutations radio-induites augmentant l'invasion et semble plutôt être régulée en fonction des constituants entourant la cellule comme par exemple la matrice extracellulaire (ECM). Plus précisément, la densité du corps tumoral et le rapprochement des cellules ainsi occasionnés sont propices à la division cellulaire alors que les cellules infiltrantes accédant facilement à la ECM auront un plus faible taux de prolifération mais une motilité augmentée grâce à ses interactions avec la EMC soit la théorie du *Go or Grow*. Un phénotype ou l'autre sera priorisé car ceux-ci sont régulés par rétro-inhibition, présentant une corrélation inverse entre la motilité cellulaire et la prolifération (Fedotov and Iomin, 2007).

La RT est anti-angiogénique de par son effet pro-apoptotique direct sur les cellules endothéliales. Par contre, une faible dose d'irradiation peut provoquer l'effet contraire, soit augmenter l'angiogenèse en induisant la libération de plusieurs facteurs pro-angiogéniques par les cellules cancéreuses comme par exemple le VEGF. Étant donné que l'angiogenèse est cruciale pour l'invasion et les métastases et que la RT peut à la fois stimuler et inhiber l'angiogenèse (Oh *et al.*, 2014), il est très important de bien comprendre la régulation entre les facteurs pro- et anti-angiogéniques pour éviter la propagation radio-induite des cellules cancéreuses par le réseau vasculaire (Madani *et al.*, 2008). De plus, connaissant le potentiel du HIF-1 à promouvoir l'invasion, l'utilisation d'agent anti-angiogéniques en même temps que la RT reste controversée. En effet, la question est toujours à l'étude à savoir si les anti-angiogéniques rendent la tumeur encore plus hypoxique, induisant ainsi l'expression de HIF-1 et rendant donc la tumeur radiorésistante par la même occasion (Barker *et al.*, 2015).

Il est maintenant bien accepté que les MMPs jouent un rôle critique dans la promotion des métastases. Une revue sur le sujet à d'ailleurs été publiée dans le journal *Nature* (Egeblad and Werb, 2002). Plusieurs études ont notamment démontré que les cellules cancéreuses peuvent sécréter des MMPs post-irradiation (Speake *et al.*, 2005; Lemay *et al.*, 2011; Chou *et al.*, 2012). Les MMPs sont aussi impliquées dans la modalité

mésenchymale du processus d'invasion. Elles dégradent les protéines de la ECM, provoquant ainsi une perte d'adhérence qui augmente la motilité des cellules cancéreuses. Par exemple, une expérience réalisée avec des tumeurs de mélanome humain implantées chez la souris, puis irradiées ensuite avec une dose sous-curative de 10 ou 15 Gy ont démontré un nombre de métastases plus élevé en comparaison avec des tumeurs non-irradiées. Ce phénomène de métastases radio-induites est associé avec de l'hypoxie au sein de la tumeur menant à une augmentation de l'expression du récepteur de l'urokinase (uPAR). La plasmine, un ligand de ce récepteur, facilite l'invasion tumorale et la promotion des métastases en activant entre autres plusieurs MMPs, qui à leur tour dégradent les composantes de la ECM (Rofstad *et al.*, 2004). Plus précisément, les MMP-2 et -9 ainsi que la métalloprotéinase de membrane de type 1 (MT1-MMP) ont souvent été associées avec une augmentation de l'invasion ainsi qu'au développement de métastases (Moncharmont *et al.*, 2014). Le groupe de Chou *et al.* a démontré *in vitro* et *in vivo* que la MMP-9 est cruciale dans le phénomène d'invasion et de métastases radio-induites dans un modèle de carcinome pulmonaire de Lewis (Chou *et al.*, 2012). En lien, la répression du gène de la MMP-9 a significativement réduit l'augmentation des métastases radio-induites de ce même modèle. En ce qui concerne la MMP-2, l'irradiation de cellules cancéreuses pancréatiques Panc-1 a provoqué une augmentation significative de l'expression *in vitro* de cette MMP, suggérant aussi son implication dans le phénomène d'invasion radio-induite du cancer (Qian *et al.*, 2002). De son côté, le groupe de Perentes *et al.* a démontré qu'une expression élevée de MT1-MMP corrèle avec une invasion des vaisseaux sanguins tel que constaté dans les spécimens de biopsies de femmes atteintes de TNBC, suggérant un risque de métastases plus élevé comparé aux autres types de cancer du sein (Perentes *et al.*, 2011). De par sa capacité à activer la MMP-2 et à dégrader les composantes de la ECM dans son environnement immédiat, la MT1-MMP est bien connue pour augmenter l'invasion des cellules cancéreuses (Sato *et al.*, 2005; Sato and Takino, 2010). Bien que peu d'études associent la MT1-MMP avec le phénomène d'invasion radio-induite, le groupe de Paquette *et al.* a mis en évidence son implication dans un modèle *in vitro* de chambre d'invasion utilisant des cellules de cancer du sein triple négatif (Paquette *et al.*, 2007). De ce fait, cette voie semble très prometteuse dans l'optimisation du traitement du cancer du sein triple négatif.

L'induction des MMPs par la radiation est bien reconnue (Annabi *et al.*, 2003), mais il faut prendre en considération que ces augmentations ne se reflètent pas toujours aussi facilement au niveau des échantillons humains post-RT encore une fois en lien avec l'hétérogénéité des cohortes (Susskind *et al.*, 2003; Moncharmont *et al.*, 2014). Des études supplémentaires sont donc nécessaires pour déterminer les mécanismes d'action précis des MMPs dans l'invasion radio-induite des cellules cancéreuses.

En bref, la radiation peut augmenter l'agressivité des cellules cancéreuses et tant et aussi longtemps que les mécanismes d'interactions de la cellule cancéreuse avec son microenvironnement tumoral ne seront pas adéquatement compris, il sera difficile de prévenir efficacement le phénomène d'invasion radio-induite.

1.2.2.2 L'invasion radio-induite par le microenvironnement

Le microenvironnement tumoral, aussi appelé stroma, se caractérise par tout autre composant du milieu tumoral qui n'est pas une cellule cancéreuse. Plus précisément, celui-ci comporte les cellules saines, les molécules libérées ainsi que les vaisseaux sanguins environnant la tumeur. Ces autres composants agissent comme niche et environnement «nourrissant» pour la tumeur (Friedl and Alexander, 2011).

La majorité des études à ce jour se concentrent sur l'effet des radiations directement sur la cellule cancéreuse (Moncharmont *et al.*, 2014), mais le rôle du microenvironnement tumoral ou stromal est de plus en plus reconnu comme étant un joueur critique dans le processus de carcinogenèse. Cette effet est d'autant plus augmenté par la radiation suite à la libération de cytokines par les fibroblastes, les péricytes, les cellules endothéliales ou encore les cellules immunitaires (Oh *et al.*, 2014). Entre autres, il a été démontré qu'une inflammation prolongée peut stimuler la migration et l'invasion tumorale. Une revue de littérature à été réalisée sur le sujet (Solinas et al., 2010) et les résultats du groupe Barcellos-hoff, 1998) ont démontrés que ces changements dans la ECM de glande mammaire irradiée peuvent persister jusqu'à plusieurs semaines post-irradiation et encourager la migration des cellules cancéreuses en facilitant leur passage dans le tissu environnant ou encore dans la circulation (Barcellos-hoff, 1998). Une dose d'irradiation

aussi faible que 0.5 à 5 Gy induit des changements rapides au niveau du stroma. Par exemple, d'autres résultats du groupe Barcellos-Hoff ont démontré l'importance du TGF-β dans ce tissu ainsi que son rôle dans la production de collagène et le remodelage de la ECM. Le stroma irradié acquière donc les caractéristiques d'un microenvironnement «dit actif» capable lui aussi de modifier le comportement des cellules environnantes. Plus précisément, le TGF-β affecte directement les fibroblastes et les transformer en myofibroblastes. Les myofibroblastes sont des fibroblastes qui expriment l'actine du muscle lisse alpha (α-SMA). Ils jouent un rôle important dans la plasticité, la migration et la motilité de la cellule au sein du tissu conjonctif, stimulant ainsi l'invasion. Par exemple, il a été démontré que des cellules cancéreuses pancréatiques en coculture avec des myofibroblastes irradiés sont beaucoup plus invasives que lorsque mélangées avec des myofibroblastes non-irradiés. Cette augmentation de l'invasion a été observée *in vitro* dans le Matrigel ainsi que dans un modèle d'implantation orthotopique *in vivo* chez la souris (Madani *et al.*, 2008). Les fibroblastes irradiés peuvent aussi libérer des molécules inflammatoires pro-invasives. De ce fait, l'irradiation de fibroblastes dans un système de coculture en chambre d'invasion avec les cellules MDA-MB-231 a permis d'augmenter de manière significative le nombre de cellules cancéreuses traversant la membrane basale de Matrigel. Cette augmentation a entre autres été associée avec une libération d'IL-1β par les fibroblastes irradiés, qui lorsqu'ajoutés au milieu conditionné de cellules non-irradiées, augmente leur invasion (Paquette *et al.*, 2013a). Cette augmentation de l'agressivité des MDA-MD-231 stimulée par les fibroblastes irradiés est prévenue par l'ajout d'un anticorps anti-IL-1β ou encore un inhibiteur sélectif à la COX-2. Cet effet supporte le rôle de l'IL-1β dans l'invasion radio-induite mais aussi son association avec la voie des prostaglandines. Un autre modèle de coculture à aussi démontré que des fibroblastes pancréatiques irradiés mélangés avec des cellules de carcinomes du pancréas enclenchent un phénotype tumoral accéléré plus agressif et plus invasif lorsque transplanté chez la souris (Ohuchida, 2004). Tous ces exemples appuient donc l'hypothèse que le stroma irradié à lui seul puisse promouvoir l'invasion des cellules cancéreuses du sein (Barcellos-Hoff, 2010) de manière TGF-β-dépendante en modifiant la ECM ou encore par la libération de cytokines pro-invasives comme IL-1β.

Le groupe de Barcellos-Hoff a aussi étudié en profondeur les effets de l'irradiation sur le stroma mammaire pour y découvrir l'importance de celui-ci dans l'apparition et la progression du cancer, mais aussi du type de cancer qui peut en émerger. Les cellules mammaires humaines COMMA-1D, ayant une tumorigénicité quasi nulle, ont été implantées chez des souris irradiées corps entier avec une dose de 4 Gy. Aussi tôt que 3 jours post-irradiation, 100% des souris ont développé des tumeurs. De plus, les tumeurs implantées chez les hôtes irradiés étaient près de 5 fois plus grosses que les quelques tumeurs observées chez les souris contrôles (Barcellos-Hoff, 2010). Bien que l'accélération de la croissance tumorale ait été associée à une activation radio-induite du TGF-β, la promotion des cancers de type basaux induits par le stroma a plutôt été associée avec les cellules souches de la glande mammaire (Barcellos-Hoff, 2010). Ce résultat confirme que le stroma irradié est un environnement propice pour la carcinogénèse.

De ce fait d'autres études ont aussi démontré que l'invasion de la tumeur peut être provoquée par le tissu irradié seulement sans même que les cellules cancéreuses n'aient été irradiées. Par exemple, l'irradiation du cerveau de rat suivi de son implantation avec les cellules tumorales de glioblastome F98 a démontré un phénotype de tumeur beaucoup plus invasif qu'une tumeur implantée dans le cerveau de rat sain. Une augmentation significative de l'infiltration des cellules tumorales dans le cerveau irradié au détriment de la croissance de la tumeur primaire a été observée (Desmarais *et al.*, 2012). Ce modèle animal a aussi mis en évidence plusieurs molécules pro-inflammatoires (IL-1β, TGF-β1, COX-2 et PGE2) ainsi que la MMP-2 comme candidats pouvant être impliqués dans ce phénomène. Un autre modèle *in vivo*, cette fois-ci chez la souris, a mené à cette même observation. En effet, la capacité d'invasion des cellules cancéreuses est augmentée de manière significative suite à l'implantation des cellules de carcinome de souris MC7-L1 ER-positives dans le muscle de la cuisse irradié. Cette fois-ci encore, les MMPs (MMP-2 et -9) ont pu être associées au phénomène d'invasion radio-induite (Lemay *et al.*, 2011).

La survie, la prolifération ou encore la radiosensibilité des cellules tumorales sont grandement influencées par l'apport en oxygène via la circulation sanguine. Les dommages au réseau vasculaire de la tumeur provoquent une réduction de cet apport en oxygène et en nutriments induisant de ce fait la mort des cellules cancéreuses par nécrose. D'un autre coté,

il est possible que le manque d'oxygène à la tumeur rende celle-ci radiorésistante (Oh *et al.*, 2014). L'environnement hypoxique post-RT induit la libération du HIF-1α et peut aussi influencer la migration. Quand l'environnement tumoral devient hypoxique, HIF-1α initie la production de facteurs angiogéniques comme VEGF et MMP-9 qui induisent à leur tour la fabrication de nouveaux vaisseaux sanguins afin de rétablir l'homéostasie. Le HIF-1α agit comme facteur de transcription chez plusieurs type cellulaires incluant les macrophages et les cellules myéloïdes progénitrices (Du *et al.*, 2008). Il est important de mentionner que les cellules inflammatoires sont elles aussi des composantes importantes du stroma qui engendrent la création d'un milieu propice à la survie, la prolifération et la migration des cellules cancéreuses par la libération de cytokines inflammatoires ou encore générer l'instabilité génomique comme discuté dans la section 1.2.1 portant sur l'inflammation radio-induite. Parfois, la fabrication de ce nouveau réseau vasculaire peut augmenter l'infiltration des cellules cancéreuses dans le parenchyme environnant, car celles-ci entrent dans la circulation sanguine (Du *et al.*, 2008). Toujours en lien avec le réseau vasculaire, des dommages aux cellules endothéliales des vaisseaux sanguins par la RT peuvent aussi augmenter le développement des métastases en facilitant l'intravasation des cellules cancéreuses (Madani *et al.*, 2008). Oh *et al.* ont isolé des cellules endothéliales dérivées de tissus mammaires humains normaux. Une dose d'irradiation de 4 Gy a permis d'augmenter la formation de tubes capillaires endothéliaux de manière ERK et MMP-2-dépendante (Oh *et al.*, 2014) et comme mentionné plus haut, la MMP-2 a contribué à l'invasion des cellules cancéreuses.

Le système immunitaire se distingue par ses deux types de défense, soit l'immunité innée et adaptative. Chacune de ces composantes peuvent aussi jouer un rôle important dans l'invasion radio-induite stimulée par le microenvironnement. Les macrophages font partie de cette première catégorie et ceux-ci sont une source majeure de production de cytokines au sein du microenvironnement tumoral (Schaue *et al.*, 2012). Il existe deux phénotypes distincts de macrophages associés aux tumeurs (TAM), soit M1 et M2. Le phénotype M1 est favorise l'inflammation en produisant une grande quantité de NOS. Le phénotype M2 tant qu'à lui, est plutôt anti-inflammatoire mais pro-angiogénique et favorise le développement de métastases. Dans un modèle animal de cancer de la prostate, l'irradiation de tumeurs de souris TRAMP-C1 avec une dose unique de 25 Gy ou une dose

de 60 Gy administrée en 15 fractions, une diminution de la vascularisation a été observée, rendant ainsi la tumeur hypoxique. Ces régions hypoxiques chroniques induites par la RT ont démontré une infiltration concentrée de TAM de phénotype M2 alors que leur distribution était de façon aléatoire au sein des tumeurs non-irradiées (Chen *et al.*, 2010). Sachant que les macrophages de phénotype M2 favorisent le développement de métastases, ceux-ci représentent un mécanisme d'action potentiel dans le phénomène d'invasion radio-induite.

Toujours en lien avec le rôle potentiel du système immunitaire dans l'invasion radio-induite par le microenvironnement, les lymphocytes T, constituants de l'immunité adaptative, peuvent aussi y jouer un rôle. Plus précisément, les lymphocytes T sont impliqués dans la reconnaissance de l'hôte et l'élimination des cellules cancéreuses. Par contre, un environnement hypoxique induit par la RT provoque une accumulation de lymphocytes T de type régulateurs au sein des organes lymphoïdes, ce qui favorise une réponse anti-autoimmune. Dans une étude réalisée par Kachikwu *et al.*, une accumulation de Treg a été observée post-irradiation de la souris (corps entier et à une tumeur de la prostate) au sein de la rate et des tumeurs. Les Treg ont depuis longtemps été suspectés comme un des mécanismes échappatoires principaux des cellules cancéreuses du système immunitaire, favorisant ainsi la progression du cancer (Kachikwu *et al.*, 2011). Les Treg libèrent aussi du TGF-β (Schaue *et al.*, 2012), qui comme discuté dans cette même section, favorise la migration des cellules cancéreuses. En résumé, l'environnement hypoxique induit par la RT favorise l'invasion radio-induite par le système immunitaire en stimulant l'accumulation de macrophages M2 ainsi que de Treg au sein de la tumeur et/ou des organes lymphoïdes.

En bref, plusieurs composants du microenvironnement tumoral incluant la ECM, les cellules immunitaires, endothéliales ou fibroblastisques subissent des modifications ou sont stimulés suite à leur exposition aux radiations ionisantes. Ultimement, ces conséquences peuvent mener à une augmentation de la carcinogenèse ou encore la progression métastatique du cancer.

1.2.3 Prévention de l'inflammation radio-induite

L'inflammation induite par la radiothérapie entraîne la libération et/ou l'expression de plusieurs molécules pro-inflammatoires. Plusieurs d'entre elles ont été associées avec un mauvais pronostic de cancer et un potentiel métastatique élevé. De plus, l'hypothèse que l'inflammation radio-induite promeut l'invasion des cellules cancéreuses a été démontrée dans plusieurs études (Mantovani *et al.*, 2008; Solinas *et al.*, 2010). Dans la pratique clinique standard, une prescription d'hydrocortisone en formulation topique est recommandée chez les patientes qui subissent de la RT afin de soulager l'érythème cutané. Bien que l'hydrocortisone réduise efficacement la réponse immunitaire et la production de cytokines inflammatoires (DrugBank, 2015), son potentiel comme inhibiteur de l'invasion radio-induite n'a peu ou pas été étudié jusqu'à maintenant. Cependant, le potentiel des anti-inflammatoires non-stéroïdiens (NSAIDs) ainsi que des inhibiteurs sélectifs de la COX-2 ont fait l'objet de plusieurs études décrivant leurs bienfaits dans la prévention du cancer et de sa progression (Ulrich *et al.*, 2006; Regulski *et al.*, 2015). Sachant que l'inflammation radio-induite est un élément crucial dans l'invasion des cellules cancéreuses, est-ce qu'un traitement anti-inflammatoire administré d'emblée avec la RT pourrait prévenir cet effet néfaste?

1.2.3.1 Anti-inflammatoires non-stéroïdiens (NSAIDs)

Le mécanisme d'action principal des NSAIDs est l'inhibition des cyclooxygénases, soit la COX-1 et la COX-2, qui ont des fonctions et des patrons d'expressions différents selon l'isoforme. COX-1 est exprimée de manière constitutive et est responsable de plusieurs fonctions homéostatiques dans les tissus comme par exemple le maintien de l'intégrité de la muqueuse gastrique, du flot sanguin rénal ou du processus de coagulation (Keskek *et al.*, 2006; Yang *et al.*, 2011). La COX-2 quant à elle est inductible en cas d'inflammation, de cancer ou dans certaines pathologies (Regulski *et al.*, 2015).

Sachant que l'inflammation a un rôle important dans le développement des cancers, il n'est pas surprenant que les NSAIDs aient été reconnus comme candidats potentiels dans la prévention de plusieurs type de cancers incluant les cancers de l'œsophage, l'estomac, la

prostate, l'ovaire, le sein et possiblement d'autres (Ulrich *et al.*, 2006). En lien, une prise d'aspirine à long terme, le NSAID de loin le plus utilisé, est associée avec une réduction de 10% du risque de développer un cancer du sein (Zhao et al., 2009). Plusieurs études ont aussi démontrées l'intérêt des NSAIDs pour minimiser les effets secondaires de la RT au niveau des tissus sains. Cet effet radioprotecteur serait notamment associé à l'arrêt du cycle cellulaire en phase G1 ou G0, évitant ainsi que les cellules se retrouvent en phases S (synthèse de l'ADN) et M (mitose), soit les phases les plus radiosensibles (Elkind, 1997). Aussi, l'inhibition de l'IL-1β par les NSAIDs (Simon et al., 1995) laisse présager son potentiel dans la prévention de l'invasion radio-induite qui est pricipalement médiée par cette cytokine.

Environ 25% des utilisateurs chroniques de NSAIDs expérimenteront de la toxicité à long terme. Les effets secondaires associés à une utilisation prolongée se manifestent par de la toxicité rénale et des saignements gastrointestinaux qui peuvent même aller jusqu'à la perforation dans les cas les plus graves. Ces complications diminuent l'intérêt d'une utilisation de routine des NSAIDs dans la prévention du cancer, car il est crucial que la balance risque/bénéfice soit favorable pour le patient (Ulrich *et al.*, 2006; Litzenburger and Brown, 2014).

1.2.3.2 Anti-inflammatoires sélectifs à la COX-2

Afin de pallier aux effets secondaires liés à l'utilisation chronique des NSAIDs, une nouvelle génération de NSAIDs a été synthétisée. À la différence des NSAIDs de première génération, les inhibiteurs sélectifs de la COX-2 n'influencent pas le métabolisme gastro-intestinal, donc préserve l'intégrité de l'estomac et limite la toxicité rénale (Regulski *et al.*, 2015).

L'expression de la COX-2 est augmentée dans 63-85% des cancers du sein de stade précoce (Regulski *et al.*, 2015). Une expression élevée de cette enzyme a aussi été répertoriée dans plusieurs autres cancers incluant le cancer colorectal, du poumon, de l'estomac, de la vessie, du pancréas, de l'endomètre ainsi que de la prostate. Sachant que cette enzyme joue un rôle dans le développement tumoral mais aussi dans la dissémination

des métastases, les propriétés anti-cancéreuses des inhibiteurs sélectifs de la COX-2 ont été largement étudiées (Greenhough *et al.*, 2009).

Plusieurs études ont mis en évidence l'augmentation de l'expression de la COX-2 post-irradiation. Parmi celles-ci, le groupe de Paquette *et al.* a investigué le potentiel des inhibiteurs sélectifs de la COX-2 dans la prévention de l'invasion radio-induite des cellules cancéreuses. Un premier modèle *in vitro* a montré l'efficacité du NS-398, un inhibiteur sélectif de la COX-2, dans la prévention de l'augmentation de l'invasion radio-induite des cellules cancéreuses du sein MDA-MB-231 stimulées par le stroma irradié. Cette étude propose un mécanisme d'invasion radio-induite COX-2/MT1-MMP-dépendant chez les cellules TNBC MDA-MB-231 et l'efficacité d'un inhibiteur sélectif de la COX-2 à prévenir ce phénomène (Paquette *et al.*, 2011). Dans un modèle *in vivo* cette fois-ci, l'utilisation du méloxicam, un autre inhibiteur sélectif de la COX-2, prévient l'infiltration radio-induite des cellules F98 de glioblastome dans un modèle de pré-irradiation du cerveau de rat en modulant l'inflammation radio-induite. Encore une fois, COX-2 et IL-1β ont été identifiés comme médiateurs clés dans l'invasion radio-induite des cellules cancéreuses et leur inhibition a prolongé significativement la survie des rats (Desmarais *et al.*, 2015).

Ces résultats supportent l'hypothèse que l'utilisation des inhibiteurs sélectifs de la COX-2, conjointement avec la RT semble être une approche très intéressante dans l'optimisation du traitement des cancers du sein agressifs (Regulski *et al.*, 2015). Par contre, des risques de complications cardiovasculaires ont été rapportés vers le milieu des années 2000, nécessitant le retrait de certains de ces inhibiteurs du marché et ralentissant encore une fois l'engouement pour de nouvelles applications cliniques pour cette classe d'inhibiteurs (Regulski *et al.*, 2015).

1.2.3.3. Chloroquine

La chloroquine (CQ) est un agent anti-malarien utilisé en clinique depuis plusieurs décennies. La CQ est reconnue pour inhiber la libération de cytokines pro-inflammatoires dans la circulation sanguine, lui conférant ainsi un intérêt dans le traitement des maladies inflammatoires chroniques comme l'arthrite rhumatoïde ou encore le lupus érythémateux

(Solomon and Lee, 2009). Même après plus de 50 ans d'utilisation, la CQ reste un médicament de choix dans le traitement et la prévention de la malaria en raison de son efficacité, sa toxicité réduite et son faible coût (Solomon and Lee, 2009). Récemment, plusieurs études ont aussi mis en évidence ses propriétés anticancéreuses (Kimura *et al.*, 2013).

Outre son effet anti-malarien, la CQ est un inhibiteur de l'autophagie. L'autophagie est un mécanisme énergétique de survie cellulaire en réponse à un stress ou un environnement défavorable pour la cellule. Celui-ci peut entre autres être induit dans les cellules cancéreuses en réponse à l'irradiation ou suite à des dommages à l'ADN (Chaachouay *et al.*, 2011). De ce fait, ce mécanisme a maintes fois été associé à une résistance aux traitements contre le cancer, incluant la RT (Chaachouay *et al.*, 2011; Kimura *et al.*, 2013).

La CQ possède une action lysosomotropique. Lorsque la CQ diffuse à travers la membrane des lysosomes, l'environnement acide de ces derniers induit une forme protonée de la molécule, l'empêchant d'en sortir. Une accumulation de CQ dans la cellule entraîne une dérégulation lysosomale et mène à un déséquilibre de plusieurs voies de signalisation (Kimura *et al.*, 2013). Entre autres, les lysosomes et le réseau trans-golgien sont responsables de la maturation de plusieurs protéines. De ce fait, il a été rapporté que la sécrétion du TGF-β est diminuée chez les souris traitées à la CQ (Basque *et al.*, 2008). La phospholipase A2 (PLA2) peut aussi être inhibée par la CQ. Cette enzyme est responsable de transformer les phospholipides membranaires en acide arachidonique, soit le principal substrat de la COX-2 pour la synthèse des prostaglandines (Solomon and Lee, 2009). Connaissant l'importance de ces facteurs au sein de l'invasion radio-induite des cellules cancéreuses, la CQ semble une drogue intéressante dans la prévention de ce phénomène.

Plusieurs études ont démontré les effets de la CQ sur l'augmentation de l'efficacité du traitement du cancer du sein et son rôle de radiosensibilisateur (Solomon and Lee, 2009; Chaachouay *et al.*, 2011). Dans une étude *in vitro*, le pré-traitement des cellules cancéreuses du sein MDA-MD-231 à la CQ a significativement réduit leur survie post-irradiation. L'augmentation de la toxicité aux cellules en combinant la CQ avec la RT s'explique entre autres par la promotion de l'apoptose radio-induite et l'augmentation de la nécrose par la CQ (Solomon and Lee, 2009). La CQ inhibe aussi l'autophagie radio-induite,

donc diminue la radiorésistance des cellules cancéreuses en bloquant un de leur mécanisme de survie. Fait intéressant, une très faible induction de l'autophagie radio-induite a été mesurée chez les cellules épithéliales mammaires HBL-100 connues comme étant moins agressives et plus radiosensibles en comparaison aux MDA-MB-231, qui sont beaucoup plus agressives (Chaachouay *et al.*, 2011)**.** Cela suggère un effet préférentiel chez les cancers agressifs. De plus, la CQ démontre une spécificité notable pour les cellules cancéreuses en comparaison aux cellules saines, préservant ainsi l'intégrité des tissus normaux. Une hypothèse qui se base sur le pH basique de la CQ, empêchant celle-ci d'entrer dans les cellules normales, a été émise comme potentiel mécanisme d'action responsable de cette protection des tissus normaux. En comparaison, l'environnement de la tumeur est généralement plus acide, facilitant l'entrée du médicament dans les cellules cancéreuses (Solomon and Lee, 2009)**.**

En bref, ces données supportent l'intérêt d'utiliser la CQ dans le traitement des cancers du sein radiorésistants comme le TNBC, principalement en lien avec son effet synergique avec la RT et son action plus efficace chez les cellules radiorésistantes. Son potentiel dans la prévention de l'invasion et la migration radio-induite reste par contre à confirmer expérimentalement.

1.3 Biomarqueurs de prédiction des effets de la radiothérapie

De toute évidence, certains patients atteints du cancer sont réfractaires au traitement de RT de par l'apparition d'une récidive locorégionale ou encore de métastases. Bien que les effets bénéfiques de la RT sur la survie soient confirmés (EBCTCG et al., 2005), l'invasion radio-induite des cellules cancéreuses est un effet secondaire potentiel grave qui peut diminuer considérablement l'efficacité de ce traitement. De ce fait, la personnalisation de la RT ainsi que la connaissance de la radiosensibilité des tissus sains et tumoraux nous permettraient de réduire les complications possibles associées à ce traitement. Plus précisément, ces paramètres sur la radiosensibilité pourraient aider à prévenir l'invasion radio-induite des cellules cancéreuses résiduelles, optimiser les régimes de RT actuels ou encore prévenir

l'irradiation sans gain thérapeutique appréciable. Bien que plusieurs molécules candidates aient été mises en évidence, aucun biomarqueur de prédiction des effets de la RT n'est actuellement utilisé de routine en clinique.

Des essais de formation de colonies *in vitro* dérivés de biopsies de peau ou encore de cellules sanguines de patients ont été mis au point afin d'individualiser les doses de RT. Malheureusement, ces essais utilisent majoritairement des fibroblastes et des lymphotes en culture, s'avérant laborieux et lents à réaliser. Les dommages à l'ADN ou les aberrations chromosomiques semblent être aussi des biomarqueurs prometteurs lorsque testés dans des cohortes de taille restreintes, mais il a été impossible d'établir une corrélation dans des cohortes plus hétérogènes (Chua and Rothkamm, 2013).

Plusieurs autres biomarquers potentiels des effets de la RT sont actuellement sous investigation. Parmi ceux-ci se retrouve les jonctions de Holliday ou encore la peroxiredoxine-I. Une haute expression des jonctions de Holliday, impliquées entre autres dans la réparation des dommages double-brins, a été associée à une sensibilité plus élevée à la RT. Ces résultats proviennent de plusieurs cohortes de patientes atteintes de cancer du sein extraites de la banque de donnée libre accès *Gene Expression Omnibus* (Hu *et al.*, 2010). Concernant la peroxiredoxine-I, cette peroxydase catalyse la réaction de réduction du H_2O_2 et de différents hydroperoxides. Les ROS induits par la RT peuvent augmenter son expression et ainsi rendre la tumeur radiorésistante (Woolston *et al.*, 2011). D'autres études de plus grande envergure ayant comme sujets l'apoptose radio-induite ainsi que la réparation des dommages doubles-brins sont aussi en cours, mais pour l'identification de biomarqueurs de radiosensibilité au niveau des tissus sains cette fois-ci (Chua and Rothkamm, 2013). Du côté des techniques plus modernes, le profil génétique des patients radiosensibles a démontré des variations génomiques avoisinnant le locus du gène TNF-α (Talbot *et al.*, 2012). Cette technique est sans doute très prometteuse, mais il est actuellement impossible d'établir le profil génétique de chaque patient avant de le traiter, pour des raisons essentiellement économiques.

Au niveau du cancer du sein, la sensibilité aux rayons ionisants varie en fonction des sous-types existants. Par exemple, les cancers estrogène-dépendants, comme ceux de type luminal A, sont particulièrement sensibles à la RT. Cela s'explique en partie par le fait

que l'estrogène accélère la transition de la phase G1 à S, ce qui laisse moins de temps à la cellule de réparer les dommages radio-induits (Kyndi et al., 2008). Une expression élevée de HER2 est aussi reconnue pour influencer la réponse à la RT en rendant la tumeur radiorésistante. L'utilisation d'anticorps monoclonaux contre ce récepteur administré conjointement avec la RT renverse ce phénomène (Pietras et al., 1999). Bien que ces cibles donnent une idée de l'efficacité du traitement de RT, il n'existe toujours aucun biomarqueur de prédiction des effets de la RT au sein de la tumeur ou des tissus sains chez les patientes atteintes de TNBC parce que ces récepteurs y sont absents.

L'IL-1β joue un rôle clé dans la progression du cancer du sein et est exprimé abondamment dans la plupart des tumeurs. Un taux élevé de cytokines incluant l'IL-1β ou le TNF-α corrèle avec un grade de tumeur et un potentiel métastatique plus élevé. Cela est entre autres associé à une induction de la EMT chez les cellules cancéreuses du sein suite à une exposition prolongée aux cytokines inflammatoires (Sullivan et al., 2009). De plus, l'IL-1β est impliquée dans l'augmentation radio-induite de l'invasion des cellules cancéreuses du sein triple négatives MDA-MB-231 (Paquette *et al.*, 2013a). Une autre étude ayant établi les profils génétiques de 157 spécimens de biopsies de cancers du sein invasifs a été réalisée par le groupe Kuo *et al.*. Cette étude a permis de mettre en évidence une signature de 45 gènes impliqués étroitement dans la régulation du système immunitaire et de l'inflammation de manière TGF-β-dépendante chez les TNBC (Kuo *et al.*, 2012). Sachant que l'IL-1β est un régulateur important en amont du TGF-β et qu'une expression élevée de cette cytokine promeut le phénomène d'invasion radio-induite, cette étude justifie la pertinence de l'IL-1β comme potentiel biomarqueur de prédiction des effets de la RT chez les TNBC (Kuo *et al.*, 2012).

La MT1-MMP joue un rôle clé dans la migration cellulaire et la progression tumorale. Dans des modèles de tumeurs expérimentaux, l'expression de la MT1-MMP chez les cellules cancéreuses ou stromales augmentent la croissance tumorale et promeut l'apparition spontanée de métastases (Perentes *et al.*, 2011). Par contre, le mécanisme précis d'initiation de la cascade métastatique en lien avec la MT1-MMP restent incompris. Au niveau clinique, une expression élevée de cette protéase mesurée dans des biopsies humaines de TNBC a été associée avec l'invasion des vaisseaux sanguins, un mauvais

pronostic et une haute incidence de métastases en comparaison avec les autres sous-types de cancer de sein (Perentes *et al.*, 2011). De plus, des lignées cellulaires de TNBC réprimées pour la MT1-MMP provoquent une diminution drastique des métastases pulmonaires lorsqu'elles sont implantées chez la souris (Perentes *et al.*, 2011). Le groupe de Paquette *et al.* a aussi mis en évidence l'implication de la MT1-MMP dans le phénomène d'invasion radio-induite stimulée par le microenvironnement irradié dans un modèle *in vitro* de TNBC (Paquette *et al.*, 2011). Bien qu'aucune étude *in vivo* ou clinique n'ait encore démontré l'implication de la MT1-MMP dans les phénomènes de migration et/ou d'invasion radio-induite, cette hypothèse stimule l'intérêt d'explorer cette avenue étant donné que la majorité des patientes atteintes de TNBC subissent de la RT. De plus, l'importance de la MT1-MMP, autant dans l'invasion des cellules cancéreuses que dans l'invasion induite par le microenvironnement a été démontrée. Cela dit, si une corrélation est observée entre la MT1-MMP et l'incidence de métastases chez les femmes TNBC ayant reçu de la RT, cette protéase pourrait représenter un biomarqueur potentiel de choix pour anticiper la réponse à ce traitement.

2. Problématique

Les décisions concernant le traitement du cancer du sein sont basées sur les paramètres histopathologiques comme le statut des ganglions, le grade histologique ainsi que la taille de la tumeur. Par contre, ces caractéristiques ne tiennent pas en compte l'hétérogénéité de la tumeur qui est d'une importance capitale dans la réponse au traitement (Martei and Matro, 2015). Il apparaît incohérent que les données associées à la tumeur ou encore à la génétique du patient soient toujours insuffisantes pour modifier les standards cliniques de RT actuels selon les sous-groupes de cancer du sein (Recht *et al.*, 2001). De ce fait, toutes les patientes sont traitées avec le même régime de RT sans savoir si la tumeur répondra positivement et aucune évaluation préalable du gain thérapeutique n'est anticipée.

3. Objectifs

Conséquemment à l'hypothèse que l'inflammation induite par la RT augmente la progression du cancer chez les patientes TNBC appuyée par l'état de l'art présenté ci-haut et aux constats auxquels elle mène, les objectifs poursuivis dans la présente thèse sont les suivants :

OBJECTIF #1. Mettre en évidence et mieux comprendre les phénomènes de migration et d'invasion radio-induite des cellules cancéreuses du sein dans un modèle de souris porteuse de carcinomes mammaires du sein triple négatifs.

OBJECTIF #2. Identifier un biomarqueur de prédiction des effets de la radiation afin d'anticiper et optimiser la réponse au traitement de RT des patientes TNBC.

OBJECTIF #1

Article 1: Induction of interleukin-1β by mouse mammary tumor irradiation promotes triple negative breast cancer cells invasion and metastasis development

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Avant-propos: J'ai participé à la conception de l'étude avec Pr Benoit Paquette. J'ai effectué toutes les expériences *in vivo*, l'imagerie et l'analyse des tissus conséquents. Hélène Therriault a réalisé les expérimentations *in vitro* et les cytokines plasmatiques ont été quantifiés par la compagnie Eve Technologies. J'ai analysé tous les résultats et j'ai rédigé la première ébauche du manuscrit. En collaboration avec les autres auteurs, j'ai participé à l'amélioration de l'article jusqu'à sa version finale.

Résumé: Il est connu qu'une inflammation soutenue peut participer au phénomène de progression du cancer. Sachant que l'irradiation de la tumeur et du microenvironnement provoque une inflammation chez la majorité des patientes, est-ce que la radiothérapie pourrait être une cause de récidive rapide chez les patientes atteintes de TNBC? Dans un modèle de souris Balb/c dans lequel des cellules triple négatives D2A1 ont été implantées, le potentiel de d'invasion radio-induite à été évalué en irradiant une tumeur mammaire avec une dose fractionnée sous-curative. L'irradiation de la tumeur a mené à une augmentation de la concentration plasmatique d'IL-1β ainsi que du nombre de cellules tumorales circulantes dans le sang, résultant ultimement à une augmentation du nombre de métastases pulmonaires chez les souris irradiées. Une augmentation de l'activité des métalloprotéinases MMP-2 et -9 a aussi été mesurée suite à l'irradiation de la tumeur. En conclusion, l'irradiation de la tumeur et de son microenvironnement induit une réponse inflammatoire qui favorise l'invasion des cellules tumorales dans un modèle murin de TNBC. Cette étude démontre l'intérêt d'un traitement anti-inflammatoire combiné avec la RT chez ce sous-groupe de patientes.

Induction of interleukin-1β by mouse mammary tumor irradiation promotes triple negative breast cancer cells invasion and metastasis development

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Abstract

Purpose: Radiotherapy increases the level of inflammatory cytokines, some of which are known to promote metastasis. In a mouse model of triple negative breast cancer (TNBC), we determined whether irradiation of the mammary tumor increases the level of key cytokines and favors the development of lung metastases.

Material and methods: D2A1 TNBC cells were implanted in the mammary glands of a Balb/c mouse and then 7 days old tumors were irradiated (4x6 Gy). The cytokines IL-1β, IL-4, IL-6, IL-10, IL-17 and MIP-2 were quantified in plasma before, midway and after irradiation. The effect of tumor irradiation on the invasion of cancer cells, and the number of circulating tumor cells (CTC) and lung metastases were also measured.

Results: TNBC tumor irradiation significantly increased the plasma level of IL-1β, which was associated with a greater number of CTC (3.5-fold) and lung metastases (2.3-fold), compared to sham-irradiated animals. Enhancement of D2A1 cell invasion in mammary gland was associated with an increase of the matrix metalloproteinases 2 and 9 activity (MMP-2, -9). The ability of IL-1β to stimulate the invasiveness of irradiated D2A1 cells was confirmed by *in vitro* invasion chamber assays.

Conclusion: Irradiation targeting a D2A1 tumor and its microenvironment increased the level of the inflammatory cytokine IL-1β and was associated with the promotion of cancer cell invasion and lung metastasis development.

Introduction

Triple negative breast cancer (TNBC) is one of the most aggressive form of breast cancer (Mahamodhossen et al., 2013; Moran, 2015). With the absence of estrogen, progesterone and human epidermal growth factor (HER2) receptors impeding selective therapy, chemo and radiotherapy (RT) remain the principal therapeutic options. Some TNBC patients benefit from RT and are cured. Conversely for others, metastases will appear during the first 3 years after treatment and cure is unlikely (Podo et al., 2010). The mechanisms responsible for the early recurrence observed in some TNBC patients are still largely unknown.

Based on the improved long-term survival following RT (EBCTCG, 2005), radiation contributes to eliminate breast cancer cells left after surgery. The treatment plan is designed to deliver the radiation dose to the resection area, and to the microfoci of cancer cells dispersed throughout the surrounding breast, and when appropriate, also to adjacent lymph nodes (Roland et al., 1985; Tortorelli et al., 2013). In some rare cases of breast cancer, RT is delivered also to reduce the initial volume of the tumor before surgery (Palta et al., 2012; Charaghvandi et al., 2015). Therefore, to assess the role of radiation in the early recurrence observed in some TNBC patients, effects on tumor cells, tumor microenvironment, and healthy tissues must be considered.

Irradiation of both tumor and healthy tissues induces an inflammatory response in most patients (Bernier et al., 2008; Bower et al., 2011). Inflammation increases the level of various cytokines and growth factors, some of which favor cancer invasion (Mcbride, 1995; Quarmby et al., 1999; Schaue et al., 2012; Mukherjee et al., 2014). Since metastases are difficult to treat and frequently fatal, identification of these inflammatory factors and a better understanding of their role could provide rationale basis for selective anti-inflammatory therapies to reduce TNBC recurrence.

In the current study, the following six inflammatory mediators that are known to stimulate cancer cell invasion or metastasis development were quantified in plasma before, midway and after irradiation of a TNBC tumor implanted in a mouse mammary gland: IL-1β (Soria et al., 2011; Paquette et al., 2013a), IL-4 (Gocheva et al., 2010), IL-6 (Liu et al., 2010), IL-10 (Sung et al., 2013) IL-17 (Du et al., 2012; Chen et al., 2013), as well as macrophage inflammatory protein-2 (MIP-2) (Kollmar et al., 2006). Among them,

the plasma level of IL-1β was significantly increased and was associated with a greater number of lung metastases, in addition to favoring the invasiveness of irradiated D2A1 cells.

Material and methods

Cell culture

Mouse breast carcinoma D2A1 cells, kindly provided by Dr. Ann F. Chambers (University of Western Ontario), derived from a spontaneous mammary tumor in a Balb/c mouse. TNBC status of the D2A1 cells was confirmed by a certified pathologist, Dr. Sameh Geha (Centre Hospitalier Universitaire de Sherbrooke), using clinical standard immunohistochemistry (IHC) protocols adapted for the detection of mouse ER, PR and HER2. Cells were maintained in a 5% CO_2 humidified incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Wisent), 2 mM glutamine, 1 mM sodium pyruvate, 100 units per mL penicillin and 100 mM streptomycin.

Invasion chamber assays

Corning BioCoat Matrigel invasion chambers (Corning, Corning, NY, USA) were rehydrated with 1 ml 0.1% bovine serum albumin (BSA) in DMEM for 2 h at 37°C. D2A1 breast cancer cells were harvested by an incubation of 10 min with 1 ml of Cell Dissociation Solution at 37°C (Thermo Fisher Scientific, Waltman, MA, USA). Cell suspension (4×10^4 cells/500 µl in DMEM 0.1% BSA) irradiated at 0 or 5 Gy were added in the upper compartment of the chamber. Cells, that have crossed the Matrigel and the porous membrane, were fixed, stained with 0.5% crystal violet and counted under the microscope. Four conditions were tested: (1) non-irradiated D2A1 cells (0 Gy), basal invasion; (2) 10 ng/ml IL-1β added to the D2A1 cells (0 Gy + IL-1β); (3) irradiated D2A1 cells (5 Gy) and, (4) irradiated D2A1 cells + IL-1β 10 ng/ml (5 Gy + IL-1β). Results were reported as the invasion ratio, relative to the basal invasion.

Cell survival clonogenic assay

One thousand D2A1 cells from a single-cell suspension were seeded into 100-mm cell culture dishes containing 10 ml of culture medium and incubated at 37^{o}C for 24 h. Cells were then irradiated with 5 Gy delivered by a ^{60}Co source (Gammacell 220, MDS Nordion). Cells were maintained in DMEM supplemented with 10% FBS during all the radiation exposures, which were performed at room temperature. After 8-10 days of incubation, the resulting colonies were fixed and stained with 0.1% crystal violet. Colonies containing more than 50 cells were counted manually to calculate the cell survival that was compared to non-irradiated cells. For each assay, three parallel samples were scored and the assays were repeated 3 times.

Implantation and irradiation of D2A1 tumors

The experimental protocol was approved by our institutional ethical committee and conformed to regulations of the Canadian Council on Animal Care (protocol # 013-14). Female retired breeder Balb/c mice (18-24 weeks old) were obtained from Charles River (Raleigh, NC, USA). FUCCI-expressing D2A1 cells (10^6/100 µl PBS), generated as previously described (Bouchard et al., 2013), were subcutaneously implanted in the third pair of mammary glands of mice anesthetized with 3% isoflurane. One week later, animals were anesthetized and then immobilized with a stereotactic mouse frame adapted to dock on the Leskell Gamma Knife® Perfexion™ (Elekta, Stockholm, Sweden). Tumors implanted in the right third mammary gland were irradiated from days 8 to 11 with 4 fractions of 6 Gy (1.33 Gy/min) at 24 h interval. Based on dosimetry performed by our institutional medical physicist team, this protocol provided a biological effective dose (BED) comparable to the standard clinical regimen of 20 x 2.25 Gy, without having to perform daily anesthesia over 20 days, which can be lethal for mice. Tumors in the left third mammary gland, which received a residual dose of < 1%, were used as non-irradiated controls. In a second group of mice (sham), animals bearing tumors in the right and left mammary glands did not receive any radiation. Tumor volumes were measured as previously described (Balin-Gauthier et al., 2006). Figure 1 summarizes the chronological order of irradiation and *in vivo* experiments.

Circulating tumor cells (CTC) quantification

Blood samples (60 µl) were collected with a micro-hematocrit capillary tube from the lateral saphenous vein of the sham (n = 3) and irradiated (n = 6) mice before (day 7 after tumor implantation), midway (day10 after tumor implantation and after 2 fractions of RT) and one week after radiation therapy (day 18). A sample volume of 5 µl was diluted 1:10 in PBS and spread on a 35 mm Petri dish. The presence of CTC in blood samples was quantified by fluorescence microscopy from 5 representative images (magnification x 100), which were acquired using the FSX100® Bio Imaging Navigator microscope (Olympus, Center Valley, PA, USA) equipped with band pass filters (Chroma Technology Corp, Bellows Falls, VT, USA) for fluorescein isothiocyanate (FITC; λex = 480/30, λem = 535/40) or tetramethylrhodamine isothiocyanate (TRITC; λex = 560/40, λem = 630/60).

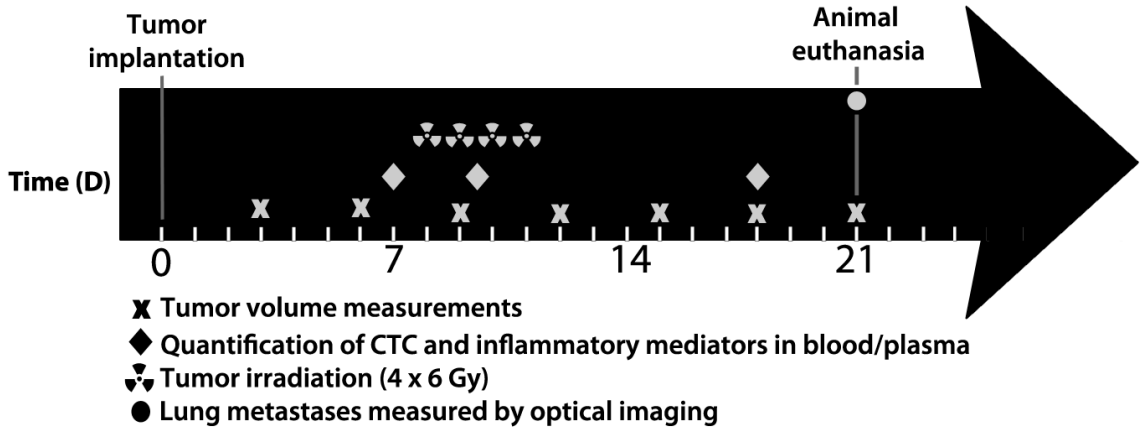

Figure 1. Study design to assess the promotion of cancer cell invasion and metastasis development after a subcurative dose of fractionated radiation delivered to a D2A1 TNBC tumor.

Cytokines quantification in plasma

Blood (50 µl) isolated from irradiated and non-irradiated mice was centrifuged at 4000g for 20 min at 4°C. Plasma samples were collected, diluted in PBS (1:2) and inflammatory cytokines IL-1β, IL-4, IL-6, IL-10, IL-17 and, MIP-2 were quantified by Multiplexing Laser Bead Technology using the Mouse Featured Cytokine 6-Plex kit (Eve Technologies, Calgary, Alberta, Canada).

Quantification of pulmonary metastases by optical imaging

Fluorescence emitted by D2A1 FUCCI-expressing cells was used to quantify the number of lung metastases with an animal optical imager (QOS® Imager, Quidd S.A.S., Val de Reuil, France). On day 21 after tumor implantation, lungs were resected from euthanized mice. A bright field image of the lungs was taken and then the appropriate filters were selected for red and green fluorescent image acquisition (mKO2, λex = 472/30, λem = 536/40; mAG, λex = 531/40, λem = 593/40). The three images acquired were merged for quantification of lung metastases.

Histology

Mammary tumors and lung specimens containing D2A1 FUCCI-expressing cells were dissected and frozen in optimal cutting temperature compound (OCT; Electron Microscopy Sciences, Hatfield, PA, USA) for cryosections or fixed with 4% paraformaldehyde (PFA) before paraffin embedding. After H&E staining, primary tumor and infiltrative areas in fixed specimen sections were delimited microscopically using Nanozoomer Digital Pathology software (Hamamatsu, Boston, MA, USA). Migration ratio was quantified as infiltrative area/primary tumor area. Cryosections of 7 µm were cut using a Leica CM3050 Microsystems cryostat (Wetzlar, Hesse, Germany). Slides were dried for 30 min at 37°C and then stored at -80°C until further use. Frozen sections of D2A1 FUCCI tumors were used to perform IHC for the detection of the CD31 blood vessel marker (sc-1506; 7 µm; dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MT1-MMP (sc-30074; 7 µm; dilution 1:100; Santa Cruz Biotechnology) and Ki67 proliferation marker (ab15580; 3 µm; dilution 1:100; Abcam Inc., Toronto, ON,

Canada) using previously described procedure (Bouchard et al., 2013). Results were expressed as percentage of CD31 stained area in the field or percentage of Ki67 positive cells. Nuclei counts were obtained automatically using the ImageJ plugin Image-based Tool for Counting Nuclei (ITCN). Staining intensity was measured according to Pham et al. method (Pham et al., 2007) adapted by Plateforme d'Analyse et de Visualization d'Images (PAVI) of the Université de Sherbrooke. For each tumor section, images of 5 to 10 representative areas were taken for quantification.

Zymogram gels

Tissues were homogenized in 150 mM NaCl, 50 mM Tris pH 7.5, 1% triton, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate buffer. MMP-2 and MMP-9 levels in tumor tissues either irradiated or control were analyzed by zymography using method previously described (Lemay et al., 2011).

Statistical analysis

Experimental data are shown as mean ± standard error mean (SEM). Statistical analyses were performed using a *t*-test and tumor growth curves analyses were performed using two-way analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered to be statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Results

Radiation therapy delays tumor growth

In clinical setting, the radiation dose used to treat breast tumors is limited by the possibility to induce undesired long term complications to normal tissues (Wazer et al., 1992). To test our hypothesis that radiation can increase the invasiveness of tumor cells that were not eliminated by the treatment, we therefore irradiated the TNBC tumor in our animal model with a dose that delays tumor growth without eliminating it completely.

D2A1 tumors were grown in the third pair of mammary glands for 7 days followed by RT being delivered only to the right tumor from days 8 to 11 after tumor implantation. Four fractions of 6 Gy were given daily for a total of 24 Gy. Another group of mice was implanted with the D2A1 cells in both mammary glands, but none of the tumors were irradiated (sham group).

Radiation significantly delayed the growth of irradiated tumors (p = 0.0234, Figure 2A). As expected, no effect was observed for non-irradiated tumors implanted in opposite mammary glands, which expanded at a similar rate then tumors implanted in sham mice (p = 0.7117, Figure 2B). These results confirm that a treatment of 4 x 6 Gy in the non-irradiated tumors implanted in opposite mammary glands can be used as sham controls in this TNBC mouse model. This model also excludes the possibility that systemic factors released by irradiated tissues influenced the growth of non-irradiated tumors.

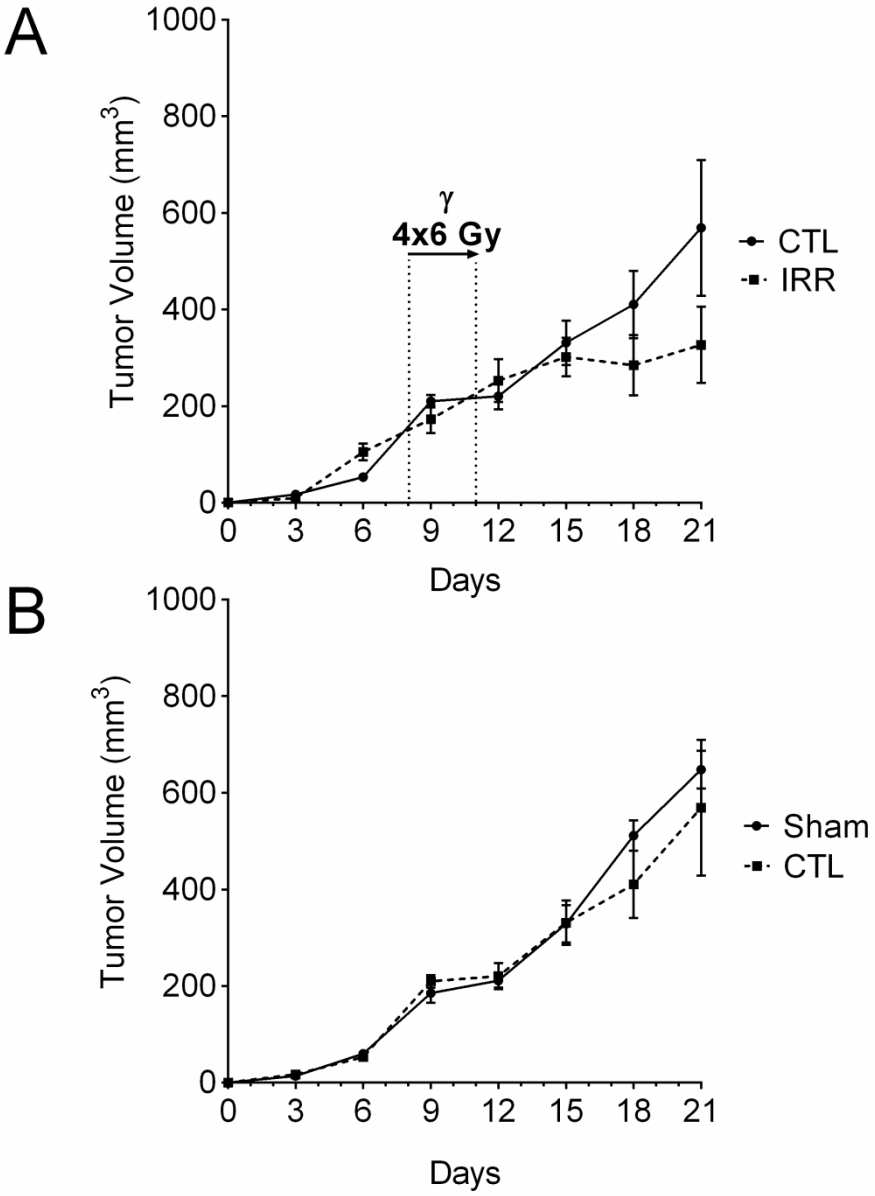

Figure 2. D2A1 tumor growth curves. **(A)** Tumor volumes were measured every 3 days after their implantation in mice mammary glands. Control (CTL) and irradiated (IRR) tumors growth curves showing a growth delay for irradiated tumors (4 x 6 Gy) compared to controls ($p = 0.0234$) ($n = 6$ tumors for each group). **(B)** Validation of the mice as their own control in irradiated animals at the right mammary tumor. D2A1 tumor volumes of sham irradiated animals (sham tumors, $n = 36$) were comparable to control tumors (left side, $n = 6$) of irradiated animals.

Tumor irradiation increases the number of CTC and lung metastases

Blood samples were collected one day before, midway and one week after tumor irradiation for FUCCI-D2A1 cells quantification by fluorescent microscopy. A significant increase of CTC (3.5-fold) was observed in irradiated mice ($p = 0.0160$), compared to the sham group (Figure 3A). One week after radiotherapy, the number of CTC in irradiated mice returned to pretreatment level, and was similar to that measured in sham mice.

Association between this CTC increase following tumor irradiation and the development of pulmonary metastases was then assessed 21 days post tumor implantation. Metastases were quantified immediately after the removal of the lungs using optical imaging. The number of lung metastases was significantly increase of (2.3-fold) in the irradiated mice compared to sham animals ($p = 0.0167$, Figure 3B and C). Stimulation of metastasis was associated with an important migration of D2A1 cells in the irradiated mammary gland, contrary to the non-irradiated tumors, where a clear demarcation with the mammary gland was visible at the tumor edge ($p = 0.0135$, Figure 4A and B). Noteworthy, a significant increase (2.7-fold) of the necrotic area was also observed in irradiated tumors ($p = 0.0233$, Figure 4C and D). Tumor vascularization and cancer cell proliferation did not appear to be related to radiation-induced migration, as no significant differences in the markers CD31 and Ki67 were found (Figure 4E and F).

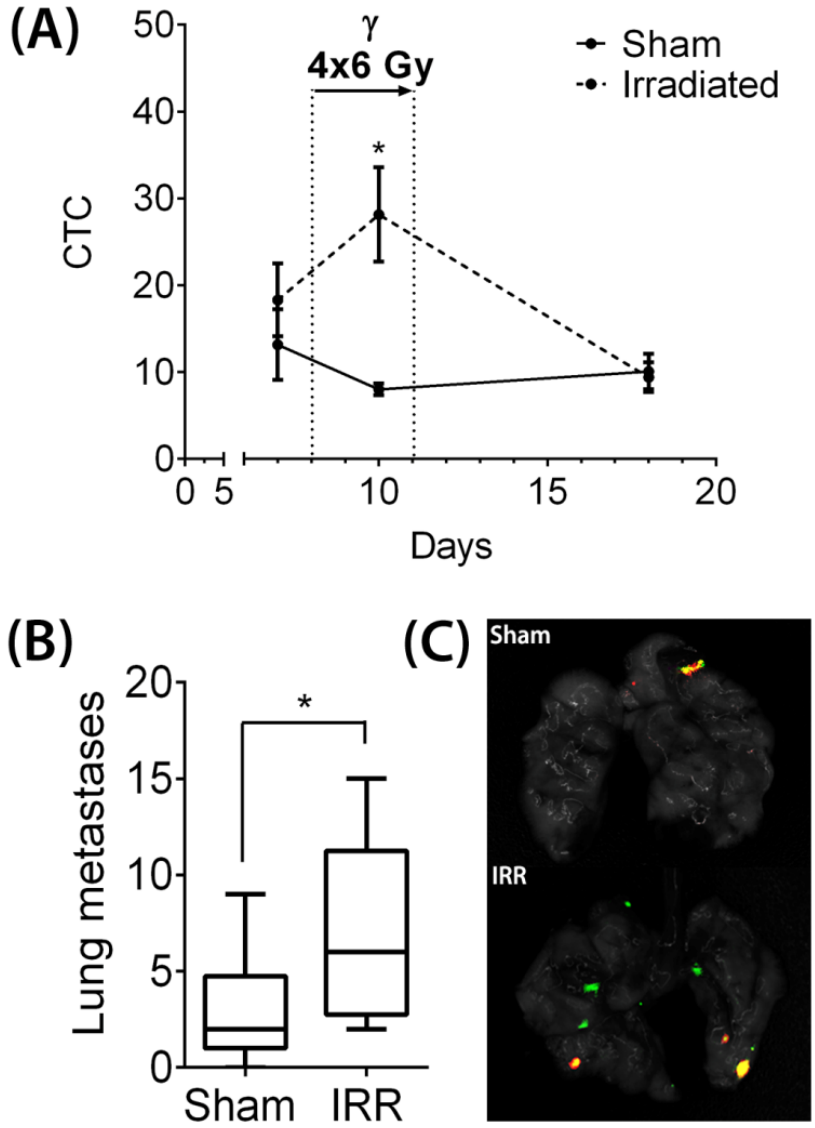

Figure 3. Radiation-induced CTC and lung metastases **(A)** Quantification of circulating tumor cells in blood samples of sham (n = 3) and irradiated mice (n = 6). CTC were significantly increased by radiation therapy compared to sham animals (p = 0.0160). **(B)** Lung metastases quantification by optical imaging. The number of metastases was increased in irradiated mice (n = 6) compared to sham group (n = 20) (p = 0.0167). Sham mice: Non-irradiated animals with tumor implantation on both sides. Irradiated animals: Irradiation of the right mammary tumor from day 8 to 11 after tumors implantation in mammary glands on both sides. **(C)** Optical imaging of lung metastases. CTC: circulating tumor cells, IRR: irradiated.

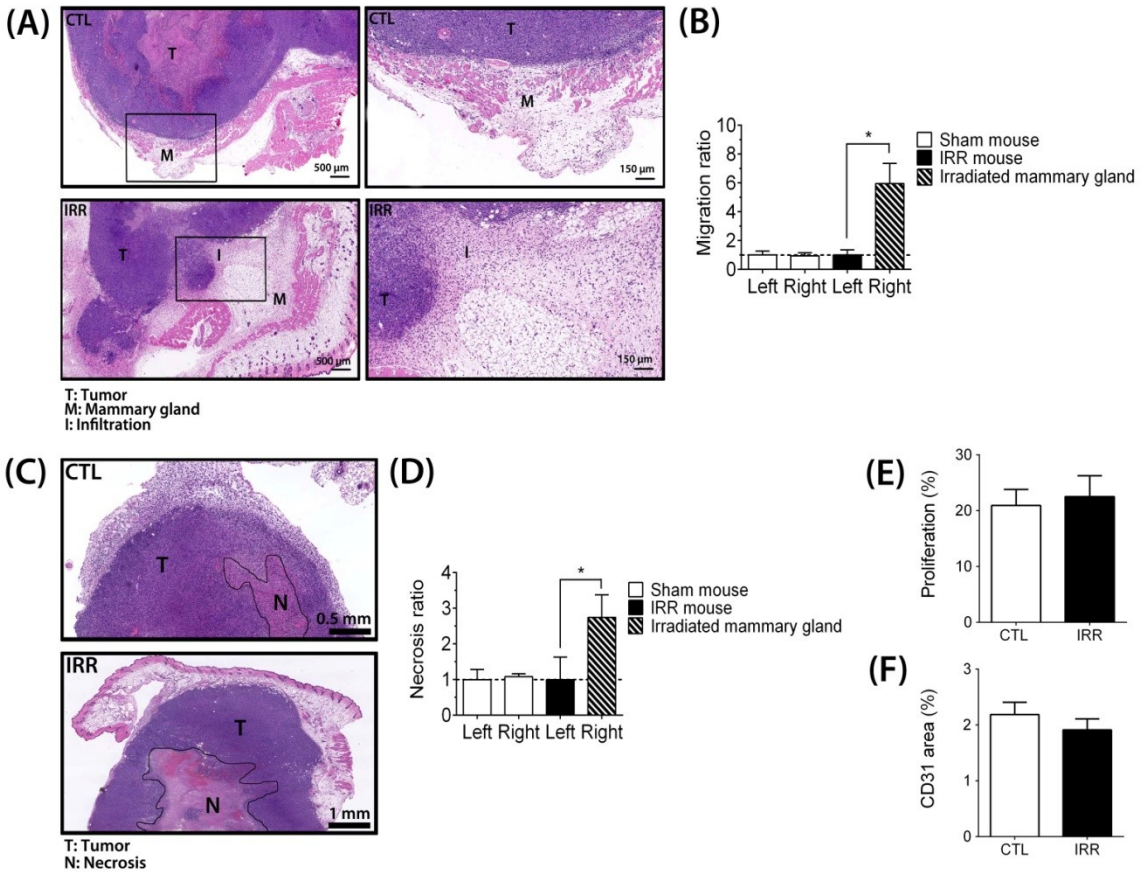

Figure 4. Radiation-induced migration in D2A1 tumors. **(A)** H&E staining from D2A1 tumor sections showing that irradiation of the tumor promotes cell infiltration into the surrounding tissue. T: tumor; MG: mammary gland; I: infiltration. **(B)** H&E quantification of tumor migration. Migration ratios were calculated as infiltrative area divided by primary tumor area. Control and irradiated tumors were compared for both groups (n = 4 to 8 H&E sections for each condition). Migration ratios were higher for irradiated animals (p = 0.0135). **(C)** H&E staining from D2A1 tumor sections showing necrotic regions of control and irradiated tumors. T: tumor; N: necrosis. **(D)** H&E quantification of tumor necrosis. Necrosis ratios were calculated as necrotic area divided by total tumor area. Control and irradiated tumors were compared for both groups (n = 2 to 5 H&E sections for each condition). Necrosis ratio were higher for irradiated animals (p = 0.0233). **(E)** Quantification of Ki67 positive cells on tumor frozen sections from control and irradiated tumors (n = 6 for each condition). No difference was observed. **(F)** Quantification of CD31 signal plotted as percentage of stained area on tumor frozen sections from control and irradiated tumors (n = 6 for each condition). No difference was observed. CTL: control, IRR: irradiated.

Tumor irradiation increases MMP-2 and -9

The proteases MMP-2 and -9 cleave extracellular matrix proteins, promoting cancer cell invasion and metastasis development (Egeblad and Werb, 2002). Induction of the activation state and protein level of these proteases in tumor by irradiation was determined by gel zymographic analyzing of tumor homogenates. A small, but significant, increase of pro-MMP-2 (p = 0.0270) and active MMP-9 levels (p = 0.0367) was measured in irradiated tumors 11 days post-irradiation (Figure 5A and B). These results support the implication of these proteases in RT-induced *in vivo* D2A1 cell migration.

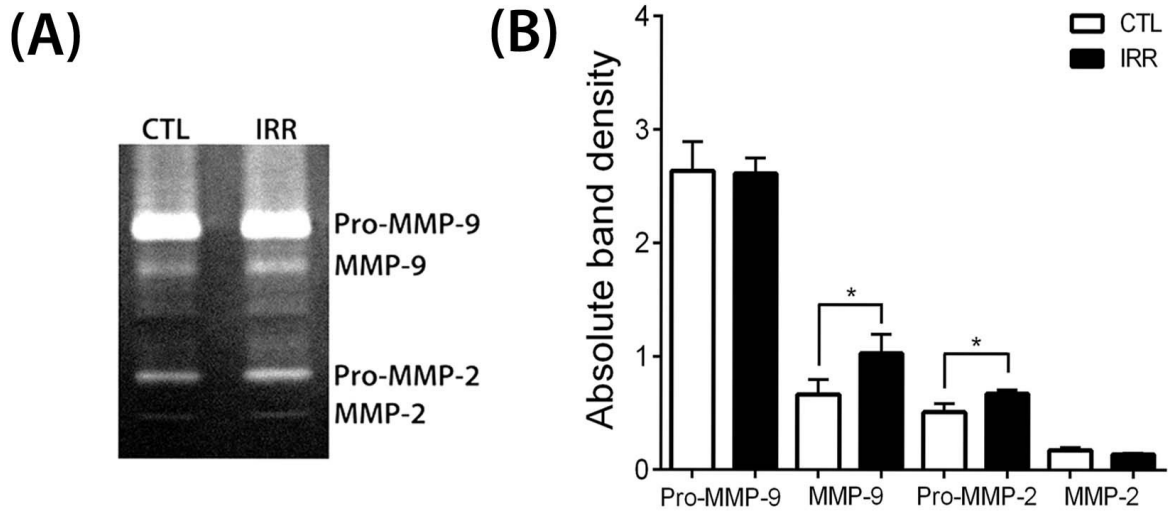

Figure 5. MMP quantification in D2A1 tumors **(A)** Zymogram gel showing a low increase of Pro-MMP-2 and active MMP-9. **(B)** Zymography quantification of the Pro and active MMP-2 and 9 band intensity using image J (n = 4 to 5 for each conditions). A light increase of Pro-MMP-2 (p = 0.0270) and active MMP-9 (p = 0.0367) were observed.

Effect of tumor irradiation on the plasma level of inflammatory cytokines

Six inflammatory cytokines, known to increase cancer cell invasion or metastasis, were quantified in plasma from sham and irradiated mice, one day before, midway and one week after tumor irradiation. Plasma samples were then analyzed using Multiplexing Laser Bead. A significant increase of plasma level was measured only for IL-1β (p = 0.0158) midway through radiotherapy (Figure 6). Plasma levels of IL-4 ($p < 0.0001$) and IL-6 (p = 0.0319) tended to increase with time, independently of radiation as no difference was observed between irradiated and sham animals. Regarding IL-10, IL-17 and MIP-2, significant difference was neither observed over time nor between sham and irradiated groups.

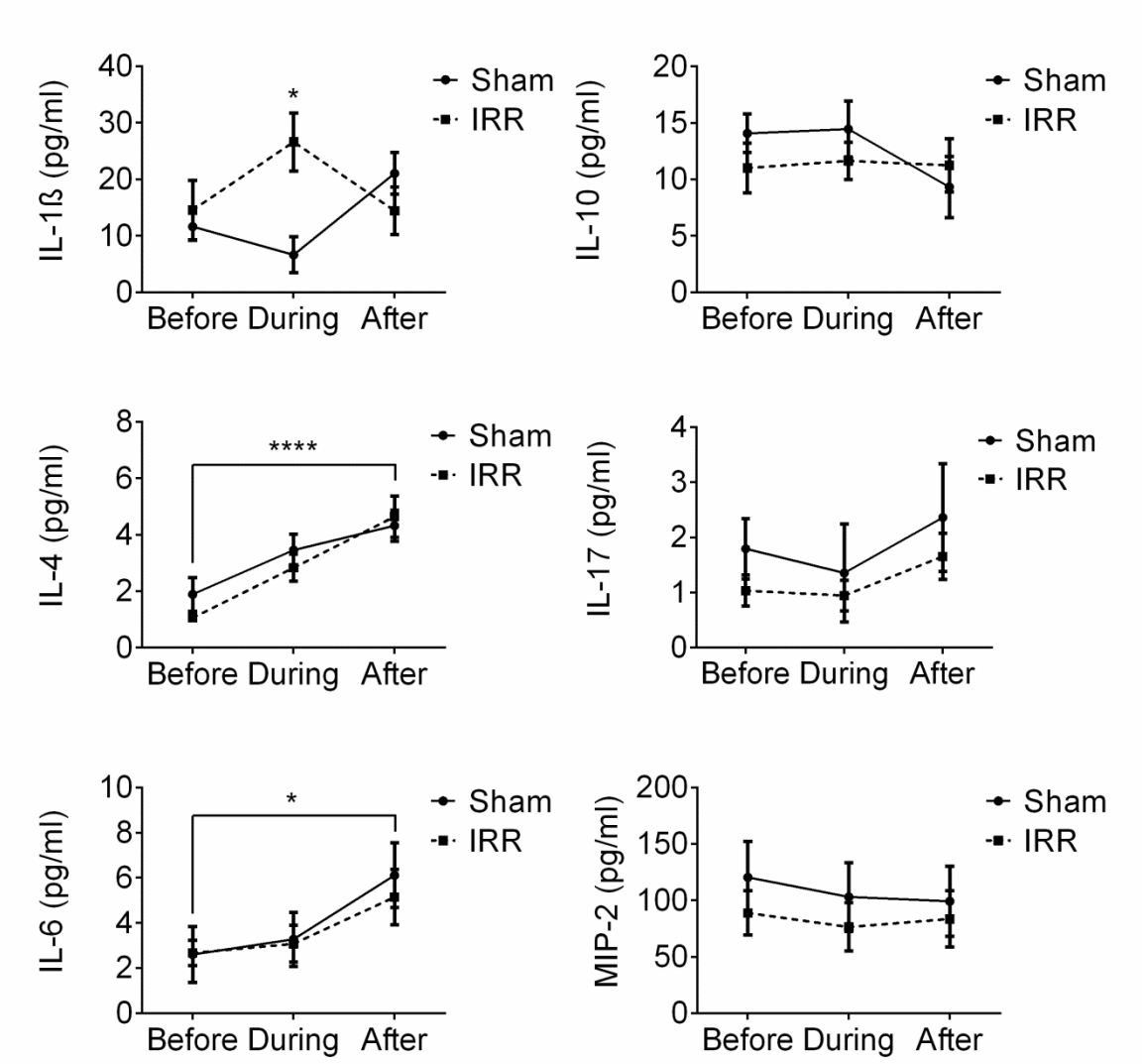

Figure 6. Plasma concentration of cytokines involved in breast cancer invasion. Cytokines were quantified 1 day before, in the middle of radiation therapy treatment (after 2 sessions) and one week after RT using Multiplexing LASER Bead Technology. IL-1β was significantly increased during RT (p = 0.0158) and IL-4 and IL-6 tend to increase during cancer progression.

IL-1β promotes D2A1 cell invasion *in vitro*

The ability of IL-1β to increase the invasion of non-irradiated cancer cells has already been reported (Lewis et al., 2006; Schaue et al., 2012; Paquette et al., 2013a). Here, we determined whether IL-1β modified the invasiveness of irradiated D2A1 cells in a Matrigel invasion chambers. Invasiveness of non-irradiated D2A1 cells was significantly

increased (1.6-fold) after the addition of IL-1β in the lower chamber compartment (p = 0.0180, Figure 7A). The survival of D2A1 cells was reduced to 8% following irradiation (5 Gy) ($p < 0.0001$, Figure 7B), resulting in a significant decrease of cell invasion *in vitro* ($p < 0.0001$, Figure 7A); but interestingly, addition of IL-1β stimulated by 2.1-fold the invasiveness of irradiated cells (p = 0.0062). These results support the potential of IL-1β to stimulate the invasiveness or irradiated cells.

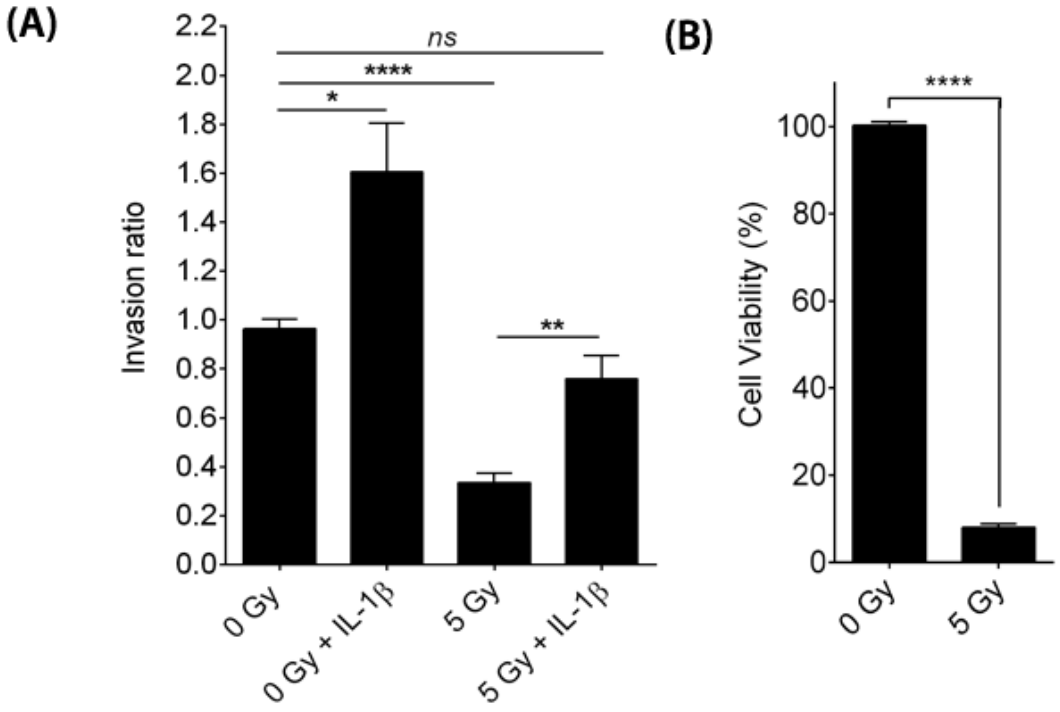

Figure 7. Role of IL-1β in the radiation-enhancement of D2A1 cell invasion. **(A)** Control (0 Gy) or irradiated (5 Gy) D2A1 cells were plated in the upper chamber in DMEM 0.1% BSA while DMEM 2% FBS with or without IL-1β (10 ng/ml) was added in the bottom chambers. Breast cancer cells that had crossed the layer of Matrigel were counted 20 h later. IL-1β significantly increased D2A1 cell invasion (p = 0.0180) and irradiation of the cells greatly prevented it ($p < 0.0001$). IL-1β restored lost invasive capabilities of D2A1 cells after irradiation (p = 0.0062). **(B)** Survival of D2A1 cells after 5 Gy irradiation as measured with a colony formation assay. n = 4 to 6 for each conditions.

Discussion

Radiotherapy has undeniably improved the survival of breast cancer patients in the last decades (Wang et al., 2011; Cancer and Objectives, 2013). However, radiotherapy might also be a two-edged sword. Toxicity to normal tissues has been usually considered the shadow of therapeutic benefit. A further rare untoward effect may be the stimulation of metastasis development by radiation. This effect, not yet completely understood, have been described in three distinct animal models (von Essen, 1991; Bouchard et al., 2013): 1) following local tumor irradiation, 2) localization of metastases in previously irradiated normal tissues, and 3) pre-irradiation of normal tissue followed by tumor implantation.

However, radiotherapy is well known to contribute to cancer cure, and there are no clinical data clearly indicating that tumor irradiation can be prometastatic. On the other hand, this effect of radiation might occur only for some subgroups of cancer, such as TNBC, but the potential role of radiation and the mechanisms involved are still largely unknown. Indeed, some TNBC patients are at high risk of early recurrence after RT and there is actually no marker available to identify these patients.

Different mechanisms were proposed to explain the radiation-enhancement of metastasis observed in animal models and some of them were established many years ago. Tumor irradiation can generate or select tumor cells more prone to form metastases (von Essen, 1991). Vascular damages induced by radiation were suggested to facilitate extravasation of tumor cells, and also to increase the number of pulmonary metastases occurring after irradiation of the lungs of mice and rats followed by i.v. injection of tumor cells (Brown and Marsa, 1978; von Essen, 1991). This effect of radiation was observed for 2 days, but not 1 week later (Withers and Milas, 1973). Pre-irradiation of normal tissue before the implantation of tumor could delay tumor growth, a phenomena called the tumor-bed-effect (TBE). It was suggested that radiation-induced damages to the host vasculature and connective tissue, resulting in impaired neovascularization, is the major cause of the TBE (Clifton and Jirtle, 1975). This retardation of tumor growth rate was associated with a larger number of metastases (Milas et al., 1988). It has also been suggested that exposure to radiation may affect the tumor stroma, particularly the wall of

the blood vessels, increasing its permeability to tumor cells, thus facilitating intravasation and metastasis (van den Brenk et al., 1977).

More recently, pre-clinical studies have revealed that inflammatory cytokines induced by pre-irradiating normal tissue can enhance cancer cell invasion, leading to a larger number of pulmonary metastases (Lemay et al., 2011; Nguyen et al., 2011; Bouchard et al., 2013). The aim of the present study was to use an animal model mimicking TNBC tumor irradiation to determine whether there is a correlation between radiation-induced inflammation and the stimulation of metastasis development. Identification of inflammatory cytokines would be an important step forward, since cancer patients could be treated with an inhibitor of these cytokines.

Our results support that IL-1β might be an important mediator of radiation-enhancement of metastasis development. Among the six inflammatory cytokines tested, only the plasma level of IL-1β was significantly increased by irradiating the TNBC D2A1 tumor implanted in the Balb/c mice mammary gland. This increase of plasma IL-1β could be associated with either irradiated microenvironment components or cancer cells, but previous studies mainly support the association of radiation-induced IL-1β by macrophages, fibroblasts and endothelial cells (Schaue et al., 2012; Isoir et al., 2013; Paquette et al., 2013a). Supporting the role of IL-1β in radiation-enhancement of cancer cell invasion, the addition of this cytokine to irradiated D2A1 cells increased their invasiveness by 2.3 fold. Other studies also reported the importance of IL-1β in the promotion of cancer cell invasion and metastasis development (Goldberg and Schwertfeger, 2010; Bower et al., 2011; Soria et al., 2011; Petrella and Vincenti, 2012; Paquette et al., 2013b; St John, 2015).

In clinical setting, IL-1β levels both in the tumor and in the serum were correlated with breast cancer invasiveness and poor prognosis (Apte and Voronov, 2002, 2008; Pantschenko et al., 2003). These results support the initiation of a clinical study to determine whether IL-1β, and also other inflammatory cytokines associated with metastasis development, are increased by irradiating TNBC patients. This study should also determine the time window during which the plasma level of inflammatory cytokines

is increased. This information would be useful to determine the posology of anti-cytokine treatment.

Plasma levels of IL-4 and IL-6 were also significantly increased over time, but this augmentation is likely to be associated with tumor growth rather than with radiation as no difference was observed between sham and irradiated animals (Magill et al., 2010; Goldstein et al., 2011; Dethlefsen et al., 2013).

IL-1β can promote tumor growth and metastasis development by inducing the expression of prometastasic genes such as matrix metalloproteinases (MMP) (Ries et al., 2007; Goldberg and Schwertfeger, 2010; Petrella and Vincenti, 2012; Petrella et al., 2012) and stimulating nearby cells to produce factors such as VEGF, IL-6, IL-8, TNF-α and TGF-β (Lewis et al., 2006). The prostaglandins pathway was also found to be indirectly increased by IL-1β in the MDA-MB-231cells (Paquette et al., 2013b).

Among others, IL-1β is largely produced by activated macrophages. Recent evidence has shown that large numbers of tumor-associated macrophages are attracted to and retained in avascular and necrotic areas, where they are exposed to tumor hypoxia (Murdoch and Lewis, 2005). In our TNBC model, tumor irradiation has greatly increased the necrotic area. Necrotic cell death releases proinflammatory signals into the surrounding tissue microenvironment which may recruit and activate inflammatory cells and stimulate angiogenesis and cancer progression (Hanahan and Weinberg, 2011). We have measured an increase of lung metastases, but no notable effect on angiogenesis was observed in irradiated tumors compared to sham tumors.

Results obtained with invasion chambers showed that radiation reduced D2A1 cell invasion, but this was associated with a massive reduction of cell survival. On the other hand, it was also reported that a sublethal dose of radiation can increase the invasiveness of some cancer cells such as pancreatic (Qian et al., 2002), glioma (Wild-bode et al., 2001; Park et al., 2006), and colon carcinoma (Wang et al., 2000; Speake et al., 2005). This promoting effect of radiation was not observed with all cancer cell lines. For example, radiation increased the invasiveness of glioma cells lacking functional PTEN (U87, U251, U373, and C6) but not those harboring wild-type PTEN (LN18 and LN428)

(Park et al., 2006). When irradiated fibroblasts were used as chemoattractant in invasion chambers, a stimulation of invasiveness were measured with the breast cancer cells D2A1, MDA-MB-231, and 4T1, and the pancreatic cancer cell lines Suit-2, Capan-1 and SW1990, but no stimulation was observed with the non-TNBC cells MCF-L1 and MCF-7 (Ohuchida, 2004; Bouchard et al., 2016).

The promotion of cancer cell invasion was associated with MMP-2 and -9 (Park et al., 2006). Down-regulation of these proteases by small interfering RNA duplexes significantly reduce the invasiveness of irradiated human glioma cell lines U251 and U87 (Badiga et al., 2011), and Lewis lung carcinoma (Chou et al., 2012). Similar reduction was also measured by adding the MMP inhibitor, CGS27023A (Qian et al., 2002).

The levels of these MMP can be enhanced by inflammatory cytokines such as IL-1β (Apte and Voronov, 2008; Paquette et al., 2013b). Our study confirmed that tumor irradiation greatly promoted the infiltration of breast cancer cells into the surrounding tissue compared to control tumors in which tumor edges were clearly delimited. This infiltration enhancement was correlated with a small but significant increase of active MMP-9 and pro-MMP-2. In our animal model, these MMP seemed to be released from the irradiated tumor cells, or infiltrated inflammatory cells such as macrophages. Indeed, the levels of these proteases were not increased in irradiated mouse mammary gland, and neither in D2A1 tumors implanted in a pre-irradiated mammary gland (Bouchard et al., 2013).

These results support that although radiation can eliminate a significant number of tumor cells, the invasiveness of some of the surviving cells might be increased by inflammatory cytokine such as IL-1β. This observation is clinically relevant because the radiation dose is optimized to remove as many tumor cells as possible, while limiting adverse effects on normal tissues. Consequently, a certain number of tumor cells may not have been eliminated by radiotherapy, and we cannot neglect that their invasiveness could be stimulated by radiation.

Also, IL-1β increases the expression of high affinity adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) on endothelial cells and by this mechanism promotes infiltration of cancer cells from the blood into tissues (Vidal-vanaclocha et al., 1994; Vidal-Vanaclocha et al., 1996; Apte and Voronov, 2008). Consequently, IL-1β facilitates lung cancer metastasis development as shown by the intravenous injection of A549 cells overexpressing IL-1β (A549/IL-1β) which are distributed to the lungs more efficiently and developed lung metastases much more rapidly than did control A549 cells. Treatment of SCID mice with anti-IL-1β antibody inhibited formation of lung metastasis by A549/IL-1β cells (Yano et al., 2003). Therefore, increasing the plasma level of IL-1β by irradiating a tumor may facilitate the extravasation of CTC resulting in a larger number of metastases.

In our animal model of TNBC, the increase of CTC did not seem to be associated with significant blood vessel damage followed by angiogenesis since no significant difference in vascularization as measured with CD31 endothelial cell marker. On the other hand, because IL-1β induces hyperpermeability in microvascular endothelium (Bohlin and Cotgreave, 1999), it is impossible to exclude that a temporary increase in vascular permeability could have facilitated the intravasation of tumor cells. Supporting this hypothesis, one week after tumor irradiation the number of CTC and IL-1β returned to their initial level, before RT. In clinical setting, an increase in the number of viable CTC has been measured after radiotherapy in non-small cell lung cancer patients (Martin et al., 2014). This result supports that radiation-enhancement of metastasis development could occur in cancer patients.

A limitation of our study is that a single cell lines was assessed. Therefore, the implication of IL-1β in radiation-stimulated metastasis development should be validated with other TNBC and non-TNBC cell lines. For *in vivo* experiments, syngeneic mouse models, as used in our study, are more appropriate since radiation-induced lung metastasis development is strongly associated with inflammation. For that reason, human xenografts implanted in immunodeficient mice would not be relevant.

In conclusion, this study has shown the importance of the tumor microenvironment, releasing pro-inflammatory cytokine IL-1β in radiation-enhancement of metastasis development. IL-1β is defined as an 'alarm cytokine' that initiates inflammatory responses, but more importantly, it induces the expression of pro-inflammatory genes (Apte and Voronov, 2008). Therefore, neutralization of a single molecule, such as IL-1β, may inhibit the generation of the cascade of downstream (effector) pro-inflammatory molecules which can promote cancer progression, especially for TNBC patients who are at higher risk of recurrence after radiotherapy.

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Declaration of interest

The authors report no conflict of interest.

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Article 2: Pre-irradiation of mouse mammary gland stimulates cancer cell migration and development of lung metastases

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Avant-propos: J'ai participé à la conception de l'étude avec Pr Benoit Paquette, Pr Caroline Saucier et Dr Rachel Bujold. J'ai effectué toutes les expériences *in vivo*, l'analyses des tissus conséquents et une partie des expériences *in vitro* en collaboration avec Hélène Therriault. J'ai analysé tous les résultats et j'ai rédigé la première ébauche du manuscrit. En collaboration avec les autres auteurs, j'ai participé à l'amélioration de l'article jusqu'à sa version finale.

Résumé: Chez la plupart des patientes atteintes du cancer du sein, la RT induit une réponse inflammatoire. Cette inflammation est en autre caractérisée par une augmentation de facteurs pro-migratoires dans le tissu sain environnant la tumeur. Cependant, leurs rôles dans la migration et la formation de métastases n'est que partiellement connu. Dans un modèle de souris BALB/c, la glande mammaire droite à été irradiée avec 4 doses de 6 Gy administrées à 24 h d'intervalle. Après la dernière séance d'irradiation, les glandes mammaires ont été récoltées pour la quantifications des molécules pro-migratoires et pro-inflammatoires ou implantées avec les cellules cancéreuses de souris D2A1 exprimant les vecteurs FUCCI. La migration des cellules cancéreuses à été suivie par imagerie optique. Au jour 21, les tumeurs et les poumons ont été récoltés pour y effectuer des analyses histologiques ainsi que le décompte des métastases pulmonaires. La pré-irradiation de la glande mammaire a augmenté significativement la migration des cellules cancéreuses, le nombre de CTC et les métastases pulmonaires. Ces effets néfastes ont été associés avec une induction de l'IL-6 et la COX-2. En général, l'apparition des métastases est associée avec une accumulation de mutations chez les cellules cancéreuses. Nos résultats suggèrent un mécanisme alternatif basé sur la libération de facteurs inflammatoires pro-migratoires dans la glande mammaire irradiée. En conclusion, l'efficacité de la RT pourrait être améliorée par des agents anti-inflammatoires, prévenant ainsi la migration radio-induite des cellules cancéreuses.

Pre-irradiation of mouse mammary gland stimulates cancer cell migration and development of lung metastases

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Running title: Pre-irradiation stimulates breast cancer progression

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Conflicts of Interest Notification: The authors report no conflict of interest.

Abstract

Background: In most patients with breast cancer, radiotherapy induces inflammation that is characterised by an increase of pro-migratory factors in healthy tissues surrounding the tumour. However, their role in the emergence of the migration phenotype and formation of metastases is still unclear.

Methods and Materials: A single mammary gland of BALB/c mice was irradiated with four doses of 6 Gy given at a 24 h interval. After the last session of irradiation, treated and control mammary glands were either collected for quantification of pro-migratory and pro-inflammatory factors or were implanted with FUCCI-expressing mouse mammary cancer D2A1 cells. The migration of cancer cells in the mammary glands was monitored by optical imaging. On day 21, mammary tumours and lungs were collected for histology analyses and the quantification of metastases.

Results: Pre-irradiation of the mammary gland increased by 1.8-fold the migration of cancer cells, by 2-fold the quantity of circulating cancer cells and by 2.4-fold the number of lung metastases. These adverse effects were associated with the induction of interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2).

Conclusion: The emergence of the metastasis phenotype is believed to be associated with the accumulation of mutations in cancer cells. Our results suggest an alternative mechanism based on pro-migratory factors from irradiated mammary glands. In clinic, the efficiency of radiotherapy could be improved by anti-inflammatory agents that would prevent the stimulation of cancer cell migration induced by radiation.

Keywords: Breast cancer, irradiation, mammary gland, metastasis, cancer cell migration.

Introduction

Radiotherapy is an important part of breast cancer treatment. This modality can completely cure the disease or eliminate a large number of cancer cells which reduces the recurrence rate and increases the overall survival of patients. It is worth noting that the total radiation dose is limited by the tolerance of surrounding normal tissues and is not meant to eradicate all cancer cells scattered in the breast, but rather to optimize long-term results with minimal adverse effects. Consequently, women still have a non-negligible risk of breast cancer death after radiotherapy (Clarke *et al*, 2005). Clinicians strive to increase the effectiveness of radiotherapy within acceptable limits of host toxicity, which aim to minimise adverse effects such as inflammation of normal tissues potentially causing fibrosis or dermatitis.

Radiotherapy is recognized to trigger an inflammatory response (Gallet *et al*, 2011). This inflammation is characterized by an increase of cytokines, angiogenic factors, adhesion molecules and matrix metalloproteinases (MMPs) (Rodemann and Blaese, 2007). It is also known that chronic inflammation increases the risk of developing several types of cancer, including breast cancer (Mantovani *et al*, 2008). Observations also suggest that radiation might promote the invasiveness of breast cancer cells (Madani *et al*, 2008). For instance, we recently reported that mouse thighs that were pre-irradiated increased the invasiveness of implanted mammary cancer cells (Lemay *et al*, 2011). Another study demonstrated that radiation promoted changes in the mammary gland stromal microenvironment that contributed to the tumourigenic potential of breast cancer cells (Barcellos-Hoff and Ravani, 2000). However, the functional roles of the pro-migratory molecules induced by radiation during the early phase of the metastatic cascade remain unresolved. A better understanding of the alleged pro-metastatic properties of radiation could contribute to the development of new therapeutic modalities to prevent these undesirable effects.

The evidence linking the microenvironment to tumour progression is growing (Goldberg and Schwertfeger, 2010). In an effort to determine the role of irradiation in the progression of breast cancer, we pre-irradiated a mouse mammary gland and then implanted triple-negative mammary carcinoma cells D2A1. This protocol allowed us to specifically assess whether inflammation induced by radiation could stimulate the progression of cancer cells. Our procedure had the advantage of defining the

mechanisms involved and eliminating confounding effects that could occur by irradiating the tumour and the mammary gland at the same time. Some examples of confounding effects are the selection of cancer cells more likely to migrate or the induction of mutations that would increase the aggressiveness of tumour cells.

While the mammary gland radiation-induced stromal effect and carcinogenesis have been studied (Barcellos-Hoff, 2010), we are, to our knowledge, the first to investigate a preclinical model of breast cancer recurrence following standard fractionated radiotherapy. Our innovative mouse model of triple-negative breast cancer cell migration is a step forward in the understanding of metastatic breast cancer.

In this study, mouse mammary glands were pre-irradiated, which stimulated the migration of mammary cancer cells at the primary site of implantation, increased the number of circulating cancer cells and promoted lung metastases. These adverse effects of radiation were associated with the increased expression in the irradiated tissue of the key pro-inflammatory factors, cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6).

Materials and Methods

Cell culture

The mouse D2A1 cancer cells, kindly provided by Dr. Ann F. Chambers (University of Western Ontario, London, ON, Canada), are derived from a spontaneous mammary tumour in a BALB/c mouse (Rak *et al*, 1992). These cells were maintained in a 5% CO_2 humidified incubator at 37^{o}C in modified Eagle's medium (MEM) (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC, Canada), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µM streptomycin.

Migration capacity of D2A1 cells assessed in invasion chambers

For the invasion assay, BALB/c 3T3 fibroblasts (2.5×10^4) were seeded with MEM supplemented with 10% FBS in 24-well plates. After 20 h, the cell culture medium was replaced with MEM supplemented with 0.1% bovine serum albumin (BSA) following two rinses in PBS. Cells were then irradiated using a ^{60}Co source (Gammacell 220, Nordion, Canada) at a dose of 5 Gy. Sham-irradiated cells were used as a control. The

fibroblast conditioned media were used as a chemoattractant in the lower compartment of the invasion chambers (Becton Dickinson Biosciences, Bedford, MA, USA). Invasion chambers coated with Matrigel (artificial basement membrane) were rehydrated with 1 ml MEM 0.1% BSA for 2 h at 37°C. Non-irradiated D2A1 mouse mammary cancer cells harvested with Cell Dissociation Solution (Sigma-Aldrich, Oakville, ON, Canada) were added (4×10^4) to the upper compartment of the invasion chambers 24 h after irradiation of the BALB/c 3T3 cells. Mouse mammary cancer cells that had passed across the Matrigel and the porous membrane 24 h later were fixed, stained with crystal violet and counted under the microscope. Results were reported as radiation-enhancement ratio. Each experiment was performed in triplicate and repeated three times.

Generation of D2A1 cells expressing the fluorescent ubiquitination-based cell cycle indicator (FUCCI) proteins

Genes encoding for the FUCCI proteins were introduced into the D2A1 cells to allow the detection and assessment of their cell cycle state by optical imaging. Replication-defective, self-inactivating CSII-EF-MCS lentiviral vectors encoding for Cdt1 and the Geminin E3 ligases substrates fused, respectively, to the red monomeric version of the Kusabira Orange (mKO2-hCdt1) and the green monomeric Azami Green (mAG-hGem) fluorescent proteins, which were generously provided by Dr. Asako Sakaue-Sawano (Brain Science Institute, RIKEN, Wako, Saitama, Japan). Red and green fluorescence are respectively markers of cells within the G1 (red) and S/G2/M (green) phases of the cell cycle (Sakaue-Sawano *et al*, 2008). Each construct was co-transfected with plasmids encoding for the lentiviral packaging proteins (plp1, plp2 and plp/VSVG) in human embryonic kidney 293T cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada). After a 48 h incubation, lentivirus-containing supernatants were harvested and filtered with a 0.45-μm filter, and then kept at −80°C until further use. D2A1 cell population expressing the FUCCI proteins were generated following a triple sequential infection for each fluorescent protein (Wu *et al*, 2010).

Mammary gland pre-irradiation and injection of D2A1 FUCCI-expressing cells

The experimental protocols were approved by the institutional ethics committee and conformed to the regulations of the Canadian Council on Animal Care. Female retired breeder BALB/c mice (18-24 weeks old) were obtained from Charles River (Raleigh, NC, USA). Animals were anesthetised with 3% isoflurane and then immobilized with a stereotactic mouse frame adapted to dock on the Leskell Gamma Knife® Perfexion™ (Elekta, Stockholm, Sweden). The third right mammary gland was irradiated by an energy deposition of elliptical shape (Figure 1A). Anesthetized mice were irradiated at a dose rate of 1.33 Gy/min to a total of 6 Gy during each of the 4 fractions at 24 h intervals. Based on dosimetry performed by our institutional medical physicist team, this protocol provided a biological effective dose (BED) comparable to the standard clinical regimen of 20 x 2.25 Gy, without having to perform daily anaesthesia over 20 days that would be lethal in mice. Regarding the non-irradiated mammary glands, they received a residual dose of less than 1%. To determine whether pre-irradiation of the mammary gland stimulated the migration of mouse mammary cancer cells, D2A1 FUCCI-expressing cells (10^6/100µl PBS) were injected 3 h after the last irradiation into the pre-irradiated (right side) and unirradiated (control, left side) mammary glands. Mouse mammary carcinoma cells were also implanted into the mammary glands of sham-irradiated mice. The tumour volume was measured every 3 days by external caliper measurements and calculated with the formula: V (mm^3) = $\pi/6$ x a (mm) x b^2 (mm^2), where "a" and "b" are the largest and smallest perpendicular tumor diameters, respectively (Balin-Gauthier *et al*, 2006). In other experiments, the D2A1 FUCCI-expressing cells (10^6/100µl PBS) were instead injected intravenously via the tail vein of sham (n=4) and pre-irradiated mice (n=4). After 9 days, these animals were euthanized and their lungs were processed to quantify the number of metastases. The number and the diameter of lung metastases were quantified using the CellProfiler 2.0.0 software. Parameters were set to an intensity-based identification method on images containing a dense amount of cancerous growth or a shape-based identification method on images containing a sparse amount of metastasis, using the fixed parameters. Figure 1B summarizes the chronological order of irradiation and the handling of animals.

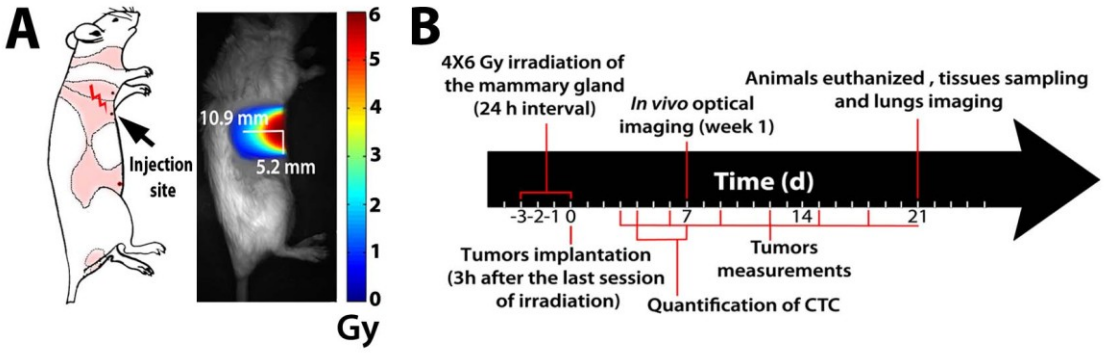

Figure 1. Description of *in vivo* irradiation model and study design. (**A**) Mouse model of irradiation, dosimetry and implantation of D2A1 cancer cells (figure adapted from Green, EL, Editor, Dover publications, 1966). (**B**) Study design for the effects of radiation on mouse mammary cancer cell migration in pre-irradiated mice mammary glands. CTC: circulating tumour cells

In vivo and *in situ* optical imaging

The migration of D2A1 FUCCI-expressing cancer cells in the mammary gland was monitored with an animal optical imager (QOS® Imager, Quidd S.A.S., Val de Reuil, France). Mice were anesthetised with ketamine/xylazine (87:13 mg/ml at 1 mg/kg). A bright field image of the mice was taken and then the appropriate filters were selected for red and green fluorescent image acquisition (mKO2, λex = 472/30, λem = 536/40; mAG, λex = 531/40, λem = 593/40). The three images acquired were merged for future analysis. Distances of D2A1 cell migration in irradiated and non-irradiated mammary glands were measured to determine the radiation-enhancement ratio. Migration was quantified with ImageJ (NIH, USA) as the distance from the nipple (physical landmark for injection site) to the end of fluorescent smear. On day 21, mice were sacrificed, and tumour and lung specimens were removed (sham; n=12 sham, irradiated; n=9). Fluorescence images of the lungs were acquired and lungs metastases were quantified as described above.

Histology

Mammary tumours and lung specimens containing D2A1 FUCCI-expressing cancer cells were collected and immediately frozen in a solution of Optimum Cutting Temperature (OCT; Electron Microscopy Sciences, Hatfield, PA, USA). Cryosections of 3 µm were

cut using a Leica CM3050 Microsystems cryostat (GmbH, Wetzlar, Germany). Slides were dried for 30 min at 37°C and then stored at -80°C until further use. The fluorescence emitted by the D2A1 cells was recorded using the FSX100® Bio Imaging Navigator microscope (Olympus, Center Valley, PA, USA) equipped with band pass filters (Chroma Technology Corp, Bellows Falls, VT, USA) for fluorescein isothiocyanate (FITC; λex = 480/30, λem = 535/40) or tetramethylrhodamine isothiocyanate (TRITC; λex = 560/40, λem = 630/60). To calculate the ratio of red-to-green fluorescence intensity of cells in the tumours, the entire slide was scanned (magnification x 42) and every image was quantified for red and green signals.

Quantification of inflammatory and pro-migratory factors

In different groups of irradiated mice (n=6 per group), animals were euthanized at 4, 7 or 24 h post-irradiation, and their mammary glands were removed and snap frozen. Prostaglandin D2 (PGD_2) and E2 (PGE_2) levels were quantified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Yang *et al*, 2002). The mRNA levels of COX-2, 15-hydroxyprostaglandin dehydrogenase (15-PGDH), IL-1β, IL-6, membrane type 1 metalloprotease (MT1-MMP), phospholipase A2 (PLA2), transforming growth factor beta 1 (TGF-β1) and tumor necrosis factor alpha (TNF-α) were determined by quantitative real-time polymerase chain reaction (qPCR) in irradiated and contralateral non-irradiated mammary glands 6 h after the last session of irradiation (n=6). Tissues were submerged in RNAlaterTM (Qiagen Inc., Toronto, ON, Canada) stored at 4°C for 24 h and then at -80°C. Total RNA extractions, reverse transcription, primer dilutions and PCR reactions were made with the FastStart Universal SYBR Green Master mix (Roche Diagnostics, QC, Canada). The following cycling conditions were used: 10 min at 95°C, and then 50 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Relative expression levels were calculated using the qBASE framework and normalized to the mouse UBC, HPRT1 and GAPDH housekeeping genes (Desmarais *et al*, 2012). The sequences of the primers that were used are listed in Table 1 in the Supplementary Materials. MMP-2 and MMP-9 levels were analyzed by zymography in mammary glands and tumour tissues in either irradiated or sham animals, using methods previously described (n=6) (Lemay *et al*, 2011) and were also confirmed by immunohistochemistry

(IHC) on 3µm paraffin-embedded tissues. MMP-2 (Thermo Scientific, IL, USA) and MMP-9 (Antibodies-Online Inc., GA, USA) antibodies signal revelation was realised using an anti-rabbit HRPO secondary antibody (dilution 1:1000; AbD Serotec, UK) and the Dako EnVision HRP system (Carpinteria, CA, USA). Tissues were counterstained with methyl-green.

Circulating tumour cells (CTC)

Blood samples were collected from the lateral saphenous vein of the sham (n=3) and pre-irradiated (n=3) mice at 4 and 7 days after the injection of D2A1 FUCCI-expressing cells in the mammary glands. Samples diluted 1:10 in PBS were spread in a Petri dish. The presence of CTC in each blood sample was quantified by fluorescence microscopy from 10 images of representative areas (magnification x 100), which were acquired as described above.

Statistical analysis

Experimental data are presented as mean ± standard error mean (s.e.m.). Statistical analyses were performed using the non-parametric Mann-Whitney test. A P value <0.05 was considered significant.

Results

Pre-irradiation of the mammary gland promotes the invasion and migration of mouse mammary cancer cells

To determine whether irradiation of the mammary gland provided a microenvironment conducive to the migration of cancer cells, we first investigated the effect of radiation on the invasion capacity of D2A1 cells *in vitro* by using invasion chambers. The BALB/c 3T3 fibroblasts were used to represent the stroma and were plated in the lower compartment of the chamber before being irradiated at 0 or 5 Gy. Our results showed that irradiated fibroblasts acted as a chemoattractant, and increased by 1.7-fold (P=0.003) the number of D2A1 cells that crossed the Matrigel layer (Figure 2A).

Then, we assessed whether pre-irradiation of mice mammary gland had an effect on the migration of D2A1 FUCCI-expressing cells by using an animal optical imager. One week after their injection close to the nipple, cells within the non-irradiated control mammary glands were forming a compact tumour at the site of implantation. In sharp contrast, in the pre-irradiated mammary glands, the D2A1 FUCCI-expressing cells had migrated away from the implantation site and were forming tumours adopting an elongated shape. The migration distance from the injection site to the front of the tumour was increased by 1.8-fold (P=0.0095) in the pre-irradiated mammary gland compared to the control non-irradiated one in the same animal (Figure 2B-C). Tumour volumes in the pre-irradiated mammary glands were also smaller (Figure 2D). This indicates that radiation favours the migration and invasion of cancer cells that occurred at the expense of tumour growth. The experiment was repeated in an independent group of mice, for whom none of the mammary glands had been irradiated. The distance of D2A1 FUCCI-expressing cell migration and growth within the mammary glands of these sham-treated mice were equivalent to those measured in the non-irradiated mammary glands of mice whose opposite mammary gland had been pre-irradiated. These results ruled out the possibility that systemic factors induced by radiation modified the migration of cancer cells implanted in the non-irradiated mammary gland. This supports the model of using a mouse in which one mammary gland is irradiated while the contralateral non-irradiated gland acts as a control, thus avoiding inter-animal variations.

Effect of radiation on cell cycle distribution

Using the animal optical imager, only red fluorescence emitted by the D2A1 FUCCI-expressing cells was observed in both sides mammary gland. This suggested that either cancer cells were concentrated in the G1 phase or the green fluorescence was attenuated by tissues (Hillman *et al*, 2011). Therefore, histological analyses were performed on frozen tumour sections that revealed a high number of red and green cells. This result supports the hypothesis that green fluorescence was attenuated by tissues. The tumour sections were then used to assess the effect of pre-irradiation of the mammary gland on the proliferation of tumour cells by quantifying cells at the G1 phase (red fluorescence) and those in S/G2/M phases (green fluorescence). Radiation increased by 26% ratio of

red-to-green cells (P=0.0356) compared to the control tumours (Figure 2E and F). The correlation between the decrease of proliferating cells (green) and the stimulation of cancer cell migration supports that pre-irradiation of the mammary gland promotes the migration of cancer cells while reducing the proliferation rate of tumour cells.

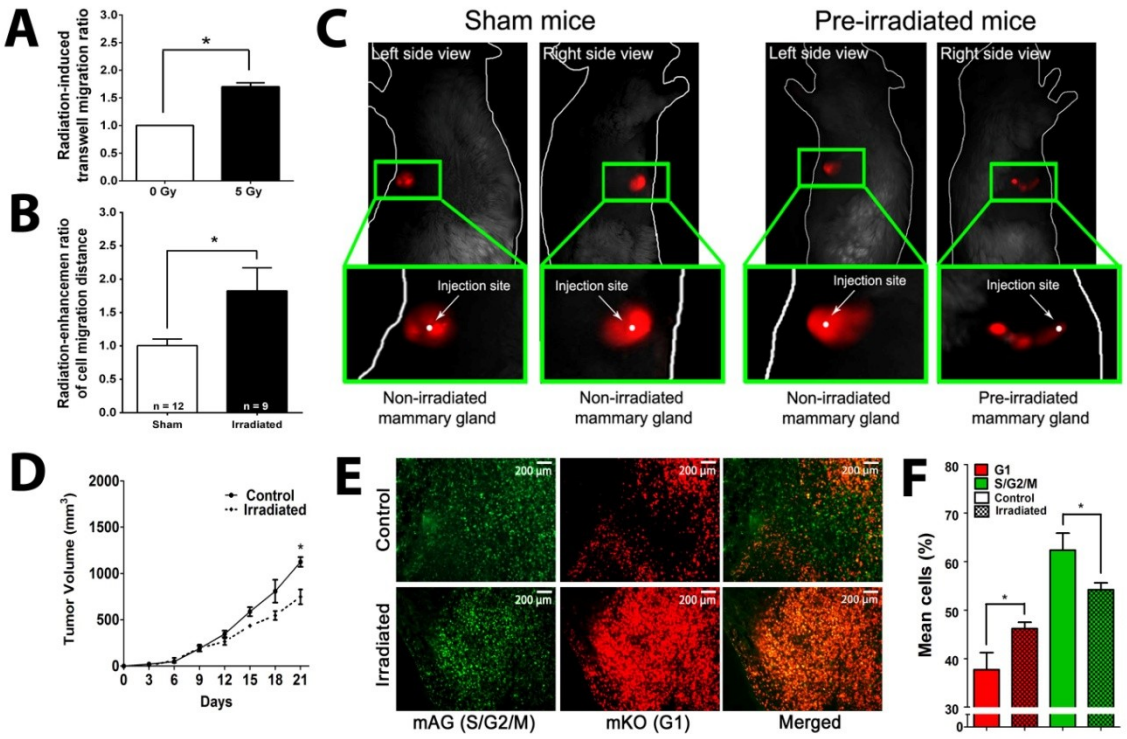

Figure 2. Migration of D2A1 FUCCI-expressing cancer cells. (**A**) *In vitro* transwell migration of D2A1 cancer cells. D2A1 migration increased by 1.7-fold (P=0.003) by irradiated (5 Gy) BALB/c 3T3 plated in the bottom chamber. (**B, C**) *In vivo* optical imaging of D2A1 FUCCI-expressing mouse mammary cancer cell migration within the mice mammary glands one week after their pre-irradiated or sham-irradiated. Animals were irradiated on the right mammary gland and the left side was used as a non-irradiated control. Tumour cells were than implanted in both sides. For the sham-irradiated mice, which had not received any radiation dose, cancer cells were implanted in the mammary glands of both sides. No radiation-induced migration in the mammary glands was observed for the sham group (n=12) compared to a 1.8-fold increase of cancer cell migration in pre-irradiated mice (n=9) (P=0.0095). Results were interpreted as radiation-enhancement ratios of cell migration in mammary glands. (**D**) Tumour volumes in pre-irradiated and non-irradiated mammary glands. (**E**) Representative fluorescence images of frozen sections of mammary tumours used to quantify cancer cells in S/G2/M (green) or G1 (red) phases. (**F**) Effect of radiation on cell cycle distribution. The mean increase of the ratio of red to green cells in pre-irradiated tissue was 26% (P=0.0356; n = 12).

Pre-irradiation of healthy mammary gland promotes lung metastases

To assess whether the stimulation of cancer cell migration induced in the pre-irradiated mammary gland affected the development of metastases, the number of lung metastases was quantified by optical imaging 21 days after the implantation of the D2A1 FUCCI-expressing cells. In the sham group, none of the mammary glands were irradiated before implantation of the D2A1 FUCCI-expressing cells on both sides. While few metastases were observed in the lungs of sham-irradiated mice, the number of metastases in pre-irradiated animals increased by 2.4-fold (P=0.0281) (Figure 3A and B). Confirming the presence of metastases, frozen sections of lungs observed under fluorescence microscopy revealed strong red and green fluorescence signals emitted by the metastases (Figure 3C). The ratio of red-to-green cells in the lung metastases was not affected whether the D2A1 cells were implanted either in the pre-irradiated or non-irradiated animals (results not shown). Supporting the hypothesis that irradiation of the mammary gland did not affect the proliferation rate of metastatic cells, the diameters of pulmonary metastases in irradiated and non-irradiated animals were not significantly different (Figure 3D). Pre-irradiation of the mammary gland increased the number of lung metastases but did not affect the metastatic cell proliferation rate.

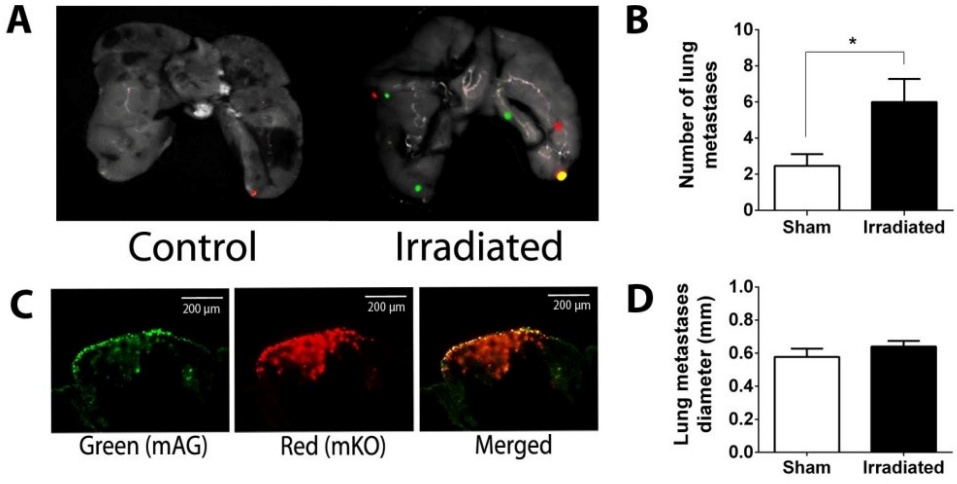

Figure 3. Pre-irradiation of the mammary gland increased the number of lung metastases. (**A, B**) *In vivo* optical imaging of lungs from irradiated and non-irradiated mice 21 days after cancer cells were implanted in the mammary gland. The number of lung metastases was 2.4 times greater in irradiated mice (P=0.0281). (**C**) Fluorescence microscopy of a pulmonary metastasis. (**D**) Mean diameter of lung metastases from irradiated and sham mice.

Mechanisms involved in radiation-enhancement of pulmonary metastases

We first hypothesized that the higher number of pulmonary metastases was caused by an increase of circulating tumour cells (CTC). CTC were easily distinguishable in blood samples and were quantified by fluorescence microscopy on days 4 and 7 after the implantation of the D2A1 FUCCI-expressing cells in the mammary glands. Mice subjected to mammary gland pre-irradiation showed a 2-fold increase in CTC on days 4 (P<0.0001) and 7 (P=0.0001) (Figure 4A).

We next verified whether pre-irradiation of the mammary gland might have released systemic factors that would favour the extravasation of circulating cancer cells to the lungs. This was assessed by directly injecting the D2A1 FUCCI-expressing cells (10^6) via the tail vein of mice with a pre-irradiated mammary gland, which were compared to a second group of non-irradiated mice. The animals were sacrificed 9 days later and their lungs removed to quantify the metastatic area by optical imaging. The number of lung metastases was not significantly different between the sham and pre-irradiated groups thus supporting that the nesting of cancer cells in the lungs was not favoured by the pre-irradiation of a mammary gland (Figure 4B).

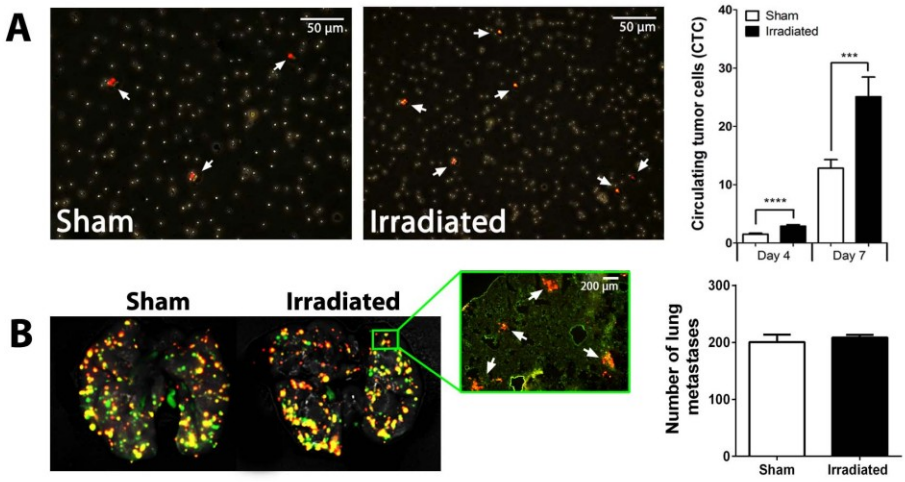

Figure 4. Mechanisms involved in radiation-enhancement of pulmonary metastases. (**A**) Blood samples were collected at the indicated time after the implantation of DA21 FUCCI-expressing cancer cells in the mammary glands of pre-irradiated and sham-irradiated mice for CTC quantification. Images were acquired by fluorescence microscopy. (**B**) Optical imaging of pulmonary metastases 9 days after tail vein injection of D2A1 FUCCI-expressing cells. The presence of cancer cells in the lungs was confirmed by fluorescence microscopy (white arrows) and the number of lung metastases in frozen lung sections was quantified in representative areas.

Assessment of pro-migratory and inflammatory factors

To characterise these adverse effects of radiation, pro-migratory and inflammatory factors were quantified in pre-irradiated mammary glands. As proteases are known to favour migration and invasion of cancer cells, the activity and/or levels of MMP-2 and MMP-9 were first determined by zymography. Surprisingly, no radiation-enhancement was observed with both MMPs in the mammary glands that were either implanted with D2A1 tumour or free of D2A1 tumours (Figure 5A and 5B), as analysed by zymography. These results were validated by IHC analyses since heterogeneous increase of MMP-2/-9 could be missed when the analysis is done in the whole mammary glands by zymography. IHC results confirmed that MMP-2 expression was not increased in irradiated and non-irradiated mammary glands free D2A1 tumour (Figure 5C-I and 5C-II). Similar levels of MMP-2 (Figure 5C-III and 5C-IV) and MMP-9 (Figure 5C-V and 5C-VI) were also obtained in tumours implanted in irradiated or non-irradiated mammary glands. The MMP-2 was specifically localised in tumour periphery with almost no expression in the tumour core. MMP-9 was moderately expressed everywhere in mammary tumours but homogeneously. Likewise, the expression of MT1-MMP, an activator of these proteases, was not stimulated by radiation (Figure 5D).

We then characterised several induced inflammatory molecules in irradiated mammary glands. The relative expression of IL-6 was significantly increased (P=0.0091), but not that of IL-1β, TGF-β1 or TNF-α, as measured by qPCR 6 h post-irradiation. Regarding the pathway of biosynthesis of PGE_2 and PGD_2, a higher expression of COX-2 was found (P=0.0039), while a modest but non-significant increase of PLA2 expression was also observed. Interestingly, 15-PGDH expression, which metabolizes PGE_2, was reduced (Figure 5D). The levels of prostaglandins PGE_2 and PGD_2, at different times post-irradiation, were quantified by LC-MS/MS. A small increase, only for PGE_2, was observed at 4 and 7 h post-irradiation (Figure 5E).

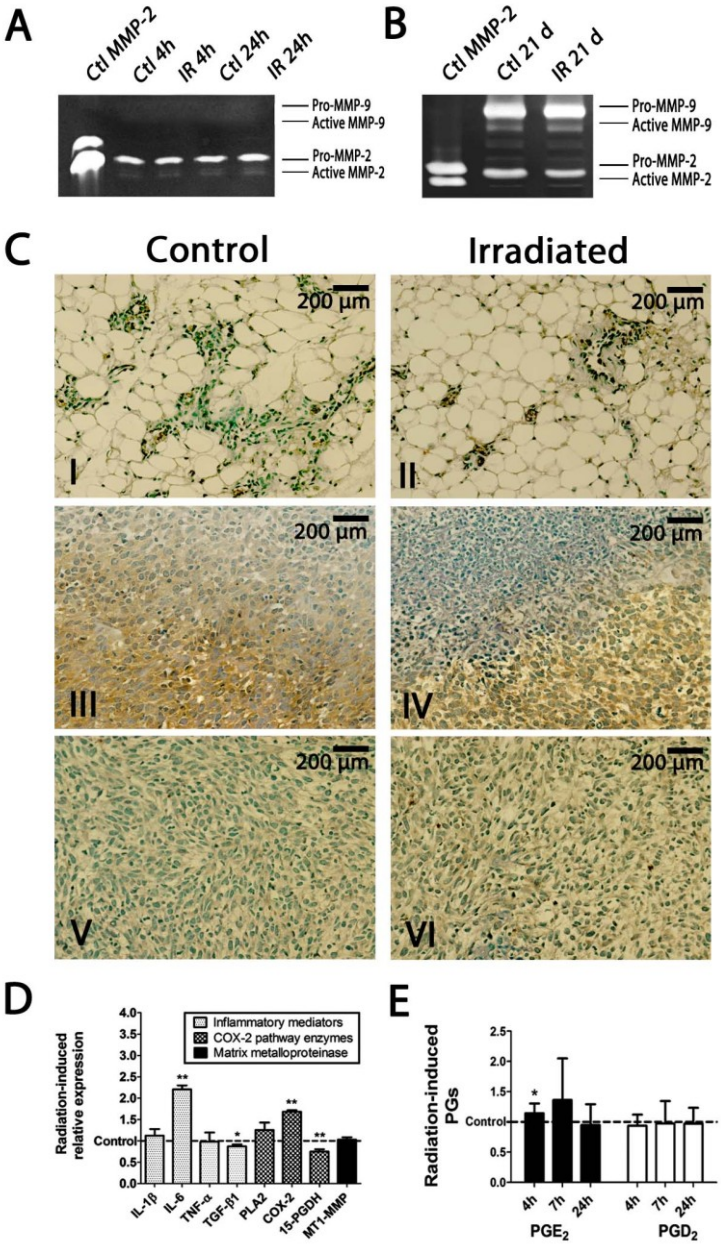

Figure 5. Radiation-enhancement of pro-migratory and inflammatory factors. (**A, B**) Zymography analyses were performed on lysates of both irradiated and non-irradiated mammary glands, which were respectively free of D2A1 cancer cells or bearing D2A1 cancer cells. MMP-2 and MMP-9 were detected in these analyses. **(C)** Immunohistochemystry of MMP-2 expression in control (I) and pre-irradiated mammary glands (II). Expression of MMP-2 (III and IV) and MMP-9 (V and VI) in D2A1 tumours implanted in pre-irradiated or not mammary glands. (**D**) The relative expression of genes potentially involved in cancer cell migration, as quantified by qPCR 6 h after the last session of irradiation. Relative mRNA expressions are plotted as a radiation enhancement ratio. (**E**) PGE_2 and PGD_2 were quantified by LCMSMS 4 h, 7 h and 24 h after the last session of irradiation. Results were plotted as radiation-induced PGs ratios calculated with an internal standard (PGE_2d^4).

Discussion

Primary breast tumours are frequently removed by conservative surgery. However, 39-63% of patients display malignant microfoci scattered throughout their breast (Holland *et al*, 1985). Therefore, protocols of radiotherapy include the whole breast and frequently a portion of the chest to include the axillary and supraclavicular lymph nodes. Consequently, a large volume of healthy tissue receives a significant radiation dose causing inflammation (Rodemann and Blaese, 2007).

The importance of the microenvironment in tumour progression is becoming increasingly accepted (Goldberg and Schwertfeger, 2010). Since inflammation can be associated with the promotion of metastases, it is important to determine whether radiation-induced inflammation in healthy breast tissue could stimulate the migration of cancer cells and ultimately favour the formation of metastases.

An enhancement of cancer cell invasion after irradiation has been reported for pancreatic cancer cells (Qian *et al*, 2002), glioma cells (Park *et al*, 2006, Wild-Bode *et al*, 2001), melanoma cells (Kaliski *et al*, 2005, Rofstad *et al*, 2004), rectal carcinoma cells (Speake *et al*, 2005) and colon carcinoma cells (Wang *et al*, 2000). These studies were designed to measure the invasiveness of irradiated cancer cells that survived after radiation treatment. The present study was designed to investigate whether irradiation of the BALB/c mouse mammary gland could stimulate the migration of mouse mammary cancer cells and the development of lung metastases. To test our hypothesis, mice mammary glands were pre-irradiated before implantation of the D2A1 mouse mammary cancer cells. This protocol eliminated confounding effects such as the selection of cancer cells more likely to migrate, which could occur by irradiating the tumour and the mammary gland at the same time.

Following irradiation of the mammary gland, a substantial stimulation of D2A1 cell migration occurred at the expense of the growth of the primary tumours, which were smaller and more elongated compared to the tumours implanted in non-irradiated mammary glands. A similar enhancement was measured *in vitro* using invasion chambers in which irradiated fibroblasts stimulated the invasiveness of non-irradiated D2A1 cells through a layer of Matrigel.

The radiation-enhancement of cancer cell migration was a local effect limited to the pre-irradiated mammary gland. Indeed, migration of the D2A1 FUCCI-expressing cells in the opposite non-irradiated mammary gland was similar to the migration found in animals who did not have any of their mammary glands irradiated. Therefore, irradiation did not seem to release pro-migratory cytokines into the circulation that would favour the migration of cancer cells in non-irradiated tissues.

The ability of an irradiated tissue to favour migration of cancer cells at the expense of growth of the primary tumour was previously reported in a glioblastoma rat model (Desmarais *et al*, 2012). Brain irradiation prior to implantation of F98 glioma cells reduced the growth of the primary tumour and favoured the infiltration of cancer cells that migrated a longer distance from the edges of the primary tumour. Notably, this switch from a proliferation to infiltration phenotype of the F98 cells reduced the mean survival time of the animals.

The emergence of a migratory phenotype is believed to be the consequence of acquired mutations in cancer cells. However, in our model, stimulation of the migratory phenotype was observed without irradiating the cancer cells. These results led us to propose an alternative explanation based on pro-migratory molecules that trigger the transition from a proliferative phenotype to an invasive one. A mutation-based hypothesis alone cannot explain the metastatic progression of all tumours. For example, a mutation-based hypothesis fails to explain the short time to recurrence of glioblastoma multiforme (GBM) after tumour resection (Hatzikirou *et al*, 2010). Giese et al. (2003) have reported that cell migration and proliferation are mutually exclusive processes for glioma cells. In their model, glioma cells proliferated only when they did not move. It turns out that the proliferation and migration of tumour cells are mutually exclusive phenotype. This mechanism, known as the migration/proliferation dichotomy (or the 'Go or Grow' mechanism), is also supported by experimental evidence showing the lower proliferation rate of migratory cells in comparison with the tumour core (Giese *et al*, 2003).

In our study with pre-irradiated mammary glands, we implanted D2A1 cells that expressed the FUCCI cell-cycle marker. This tool allowed us to confirm a transition to the G1 phase and a depletion of the S/G2/M phases in the tumour cells implanted in pre-

irradiated mammary glands, thus supporting a transition from the proliferative to migratory phenotypes.

Cell migration is a coordinated process, and it is likely that changes in the expression of several genes are required for cancer cells to become mobile. Carcinomas can undergo an epithelial to mesenchymal transition (EMT) and then move through a matrix-filled space by using proteases (Nabeshima *et al*, 2002). TGF-β1 can increase the migration of cancer cells by inducing an EMT (Romagnoli *et al*, 2012). In the pre-irradiated mammary glands of BALB/c mice, TGF-β1 gene expression was not increased. A similar result was reported by Barcellos-Hoff et al. who described an increase of the activation of latent TGF-β1 by radiation rather than an elevation of gene expression (Barcellos-Hoff, 1993, Barcellos-Hoff *et al*, 1994). Moreover, inflammation is a very dynamic process. Our specific time point may have missed TGF- β1gene expression, as well as any other inflammatory mediators that were not reported to be increased by radiation in this study.

However, the majority of solid tumours do not undergo an EMT (Sahai, 2005). These cancer cells migrate by adopting an amoebid style of movement, which does not require proteases because the cells are able to squeeze through gaps in the extracellular matrix (ECM) (Sahai, 2005). By using *in vivo* videomicroscopy, it was previously reported that D2A1 cells in mouse liver can squeeze through hepatocytes (Morris *et al*, 1994).

Levels of the MMP-2 and MMP-9 proteases were not significantly increased in the pre-irradiated mammary gland, nor in the D2A1 tumours. Nevertheless, MMP-2 was probably helping the migration of cancer cells since a high expression of this protease was found in the mammary glands. Supporting a potential role of MMP-2 in tumour progression, the IHC analyses demonstrated that MMP-2 was expressed exclusively in tumour periphery. We cannot also rule out the role for MMP-9 and the MMP activator MT1-MMP in radiation-induced migration because it was reported that radiotherapy increased by 2 to 18-fold the plasma level of MMP-9 in women with breast cancer (Riekki *et al*, 2000). We propose, in our animal model, that the increase of D2A1 cancer cell migration would be associated with amoeboid-like movement and MMP-2.

Therefore, whether MMPs inhibitors could have a beneficial role in the prevention of the radiation-enhancement of metastasis remains to be assessed.

Extravasation of circulating cancer cells to organs is in part reliant on the expression of adhesion molecules like the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the surface of endothelial cells. Their expression can be stimulated by IL-1β and TNF-α, but not by IL-6 (ten Kate *et al*, 2006). In our mouse model, only the expression of IL-6 was promoted by radiation. The extravasation rate of circulating cancer cells did not seem to be affected by pre-irradiation of the mammary gland, since intravenous injection of D2A1 FUCCI-expressing cells in the tail vein led to a similar number of lung metastases in the pre-irradiated and non-irradiated animals. However, the stimulation of lung metastases induced by radiation was associated with an elevation of CTC. The mechanisms responsible for this enhancement of CTC induced by pre-irradiation of the mammary gland are unclear. The increase in CTC in our model does not seem to be attributed to a stimulation of angiogenesis within the mammary gland. Vascular endothelial growth factor (VEGFA) expression within the mammary gland, as assessed by qPCR (supplementary Figure 1A), was not enhanced 6 h after irradiation. The number of blood vessels did not increase either, which were quantified by immunohistochemistry with the CD31 endothelial cell marker within the tumour-bearing pre-irradiated mammary gland (Supplementary Figure 1B). However, whether pre-irradiation might, by increasing inflammatory cytokines, promote vascular permeability or damage to the basement membrane within the mammary gland (thereby facilitating access of the cancer cells to the circulation) will require further investigation.

COX-2 is a key enzyme in the inflammatory response that mainly produces PGE_2. Notably, elevated expression of COX-2 in human breast cancer biopsies has been associated with distant metastases and poor prognoses (Ranger *et al*, 2004, Zerkowski *et al*, 2007). While COX-2 is known to be up-regulated by radiation (Yang *et al*, 2011), its inhibition was shown to decrease tumour growth, angiogenesis and metastasis in breast cancer mouse models (Chang *et al*, 2004, Greenhough *et al*, 2009, Tian and Schiemann, 2010). To counterbalance COX-2, PGE_2 is degraded by 15-PGDH. Interestingly, Wolf et al. showed that a low level of 15-PGDH was found in highly metastatic breast carcinoma MDA-MB-231 cells and an up-regulation of 15-PGDH significantly decreased their

ability to form tumours in athymic mice (Wolf *et al*, 2006). Our study supports such a role for COX-2 and PGE_2 since a stimulation of PGE_2 and a reduction of 15-PGDH were concurrently associated with the promotion of cancer cell migration and lung metastases. We have also previously shown *in vitro* that PGE_2 enhanced breast cancer cell invasion, while COX-2 inhibitor prevented radiation-enhancement of breast cancer cell invasion (Paquette *et al*, 2011). Therefore, it would be interesting in future *in vivo* studies to evaluate whether the use of COX-2 inhibitors might represent an efficient way to prevent radiation-induced lung metastases.

In conclusion, we have shown in the current study that pre-irradiation of the mammary gland increased the migration of mouse mammary cancer cells, the quantity of circulating cancer cells and the number of lung metastases (Figure 6). These adverse effects were not due to mutations induced by radiation in cancer cells, but rather to pro-migratory molecules induced in the microenvironment of irradiated mammary glands. On the other hand, we cannot exclude that vascular and microenvironment changes occurring during tumour growth could also contribute to the migration of cancer cells after irradiation. In clinic, our results might suggest that the efficiency of radiotherapy could be improved by preventing the stimulation of cancer cell migration induced by radiation

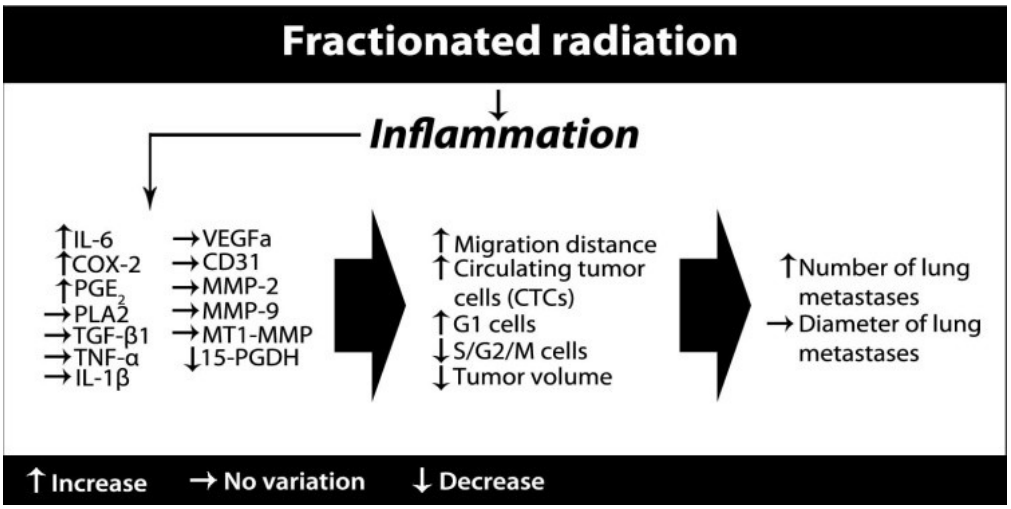

Figure 6. Model of breast cancer cell migration stimulated by radiation. We propose that inflammation mediated by fractionated radiation in breast cancer therapy causes a substantial enhancement of cell migration and CTC-promoting pulmonary metastases.

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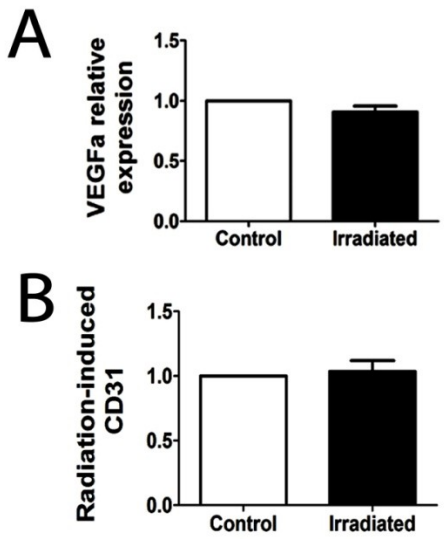
Supplementary material

Supplementary methods

Immunohistochemistry (IHC) was performed on D2A1 FUCCI tumors for the detection of the CD31 blood vessel marker (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signal revelation was realized using an anti-goat HRPO secondary antibody (dilution 1:3000; Cedarlane, Burlington, ON, Canada) and the Dako EnVision HRP system (Carpinteria, CA, USA). Tissues were counterstained with methyl-green. For each tumor, images of 10 representative areas of 3 serial sections were taken at 20X magnification for signal quantification. The results were expressed as percentage of CD31 stained area in the field. VEGFa was quantified by relative qPCR as previously described in methods and material.



Supplementary Figure 1. Effect of radiation on mammary gland and tumor vascularization (**A**) The relative expression of VEGFa in irradiated mammary gland was quantified by qPCR 6h post-irradiation. (**B**) The level of CD31 was assessed in D2A1 tumor implanted in pre-irradiated and control mammary glands by IHC.

Table 1 Quantitative polymerase chain reaction (qPCR) primers sequences

Genes	Forward name	Reverse name	Sense primers	Antisense primers
15-PGDH	Hpgd.q.F1	Hpgd.q.R1	5'- GGATATTTTGGTCAACAATGCAGGCG-3'	5'- CCTTCACCTCCGTTTTGCTTACTCA-3'
IL-1β	Il1b_G_1_f	Il1b_G_1_r	5'- TAGCCCGCACTGAGGTCTTT-3'	5'- AGCAATGTGCTGGTGCTTCA-3'
MT1-MMP	Mmp14.q.F1	Mmp14.q.R1	5'- CCCTCGCTGTGGTGTTCCG-3'	5'- TGTGGCATACTCGCCCACCTTA-3'
PLA_2	Pla2g4a.qmm.F1	Pla2g4a.qmm.R2	5'- GCTCCCGACTGAGAGCCAGAA-3'	5'- CCAGTTGCAGAAATTCTCACACTTCC-3'
COX-2	Ptgs2_G_f	Ptgs2_G_r	5'-TGGTTTTGTGCTGGCCTGGTA-3'	5'-TTCGAAGTTCAGCCTGGCAAGT-3
TGF-β1	Tgfb1_G_2_f	Tgfb1_G_2_r	5'- GCGGCAGCTGTACATTGACTTT-3'	5'- AGAAGTTGGCATGGTAGCCCTT-3'
IL-6	IL-6.F1	IL-6.R1	5'-ACAAGTCGGAGGCTTAATTACACAT-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
TNF-α	TNF-alpha.F1	TNF-alpha.R1	5'-AGGCGGTGCCTATGTCTCA-3'	5'-GGGTCTGGGCCATAGAACTG-3'
VEGFa	Vegfa.qmm.F1	Vegfa.qmm.R1	5'-CCCACGACAGAAGGAGAGCAGA-3'	5'-CACACAGGACGGCTTGAAGATGT-3'
References				
GAPDH	GAPDH.mouse.fwd1	GAPDH.mouse.rev	5'-TGACGTGCCGCCTGGAGAAA-3'	5'-AGTGTAGCCCAAGATGCCCTTCAG-3'
HPRT1	HPRT1.mouse.fwd1	HPRT1.mouse.rev1	5'-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3'	5'-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3'
UBC	UBC.mouse.fwd1	UBC.mouse.rev1	5'-CGTCGAGCCCAGTGTTACCACCAAGAAGG-3'	5' CCCCCATCACACCCAAGAACAAGCACAAG-3'

15-PGDH: 15-Deoxyprostagladin dehydrogenase, IL-1β: Interleukin-1 beta, MT1-MMP: Matrix metalloproteinase type 1, PLA_2: Phospholipase A2, COX-2: Cyclooxygenase 2, TGF-β1: Transforming growth factor beta 1, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, HPRT1: Hypoxanthine phosphoribosyltransferase 1**,** UBC: Ubiquitin C**,** IL-6: Interleukin-6, TNF-α: Tumor necrosis factor alpha, VEGFa: Vascular endothelial growth factor

Article 3: Stimulation of triple negative breast cancer cell migration and metastases formation is prevented by chloroquine in a pre-irradiated mouse model

Auteurs: Gina Bouchard, Hélène Therriault, Sameh Geha, Yves Bérubé-Lauzière, Rachel Bujold, Caroline Saucier and Benoit Paquette

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Avant-propos: J'ai participé à la conception de l'étude avec Pr Benoit Paquette. J'ai effectué toutes les expériences *in vivo*, l'imagerie et l'analyse des tissus conséquents. Hélène Therriault a réalisé les expérimentations *in vitro*. J'ai analysé tous les résultats et j'ai rédigé la première ébauche du manuscrit. En collaboration avec les autres auteurs, j'ai participé à l'amélioration de l'article jusqu'à sa version finale.

Résumé: Certaines patientes atteintes de TNBC ont un risque de récidive plus élevé dans les trois premières années suivant le traitement. Cette récidive rapide est en partie associée à une réponse inflammatoire radio-induite dans les tissus sains qui favorisent la migration des cellules cancéreuses ainsi que la formation de métastases. Dans cette étude, le potentiel de la CQ à inhiber le développement des métastases pulmonaires radio-induites a été étudié. Une seule glande mammaire du côté droit de la souris a été irradiée avec 4 doses de 6 Gy à raison de 24 h d'intervalle. Après la dernière séance d'irradiation, les glandes mammaires contrôles et irradiées ont été implantées avec les cellules de souris triple négatives D2A1. Un traitement de CQ a été administré par injection intra-péritonéale (saline, 40 ou 60 mg/kg) 3 h avant chaque séance d'irradiation puis à chaque 72 h pendant les 3 semaines suivantes. Nos résultats démontrent que la CQ prévient la migration des cellules D2A1dans la glande mammaire de souris ainsi que l'augmentation radio-induite des métastases pulmonaires. Cet effet protecteur de la CQ est associé à une réduction de l'expression des facteurs inflammatoires IL-1β, IL-6 et COX-2, mais le niveau d'expression des MMP-2 et -9 n'a pas été modifié. La CQ a aussi induit une blocage de l'autophagie, soit un mécanisme de survie cellulaire en réponse à un stress. En conclusion, la CQ peut prévenir l'augmentation radio-induite des cellules cancéreuses ainsi que des métastases pulmonaires dans un modèle de souris TNBC. Cette étude suggère l'intérêt d'un traitement anti-inflammatoire combiné avec la radiothérapie chez ce sous-groupe de patientes.

Stimulation of triple negative breast cancer cell migration and metastases formation is prevented by chloroquine in a pre-irradiated mouse model

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Abstract

Background: Some triple negative breast cancer (TNBC) patients are at higher risk of recurrence in the first three years after treatment. This rapid relapse has been suggested to

be associated with inflammatory mediators induced by radiation in healthy tissues that stimulate cancer cell migration and metastasis formation. In this study, the ability of chloroquine (CQ) to inhibit radiation-stimulated development of metastasis was assessed.

Methods: The capacity of CQ to prevent radiation-enhancement of cancer cell invasion was assessed *in vitro* with the TNBC cell lines D2A1, 4T1 and MDA-MB-231 and the non-TNBC cell lines MC7-L1, and MCF-7. In Balb/c mice, a single mammary gland was irradiated with four daily doses of 6 Gy. After the last irradiation, irradiated and control mammary glands were implanted with D2A1 cells. Mice were treated with CQ (vehicle, 40 or 60 mg/kg) 3 h before each irradiation and then every 72 h for 3 weeks. Migration of D2A1 cells in the mammary gland, the number of circulating tumor cells and lung metastasis were quantified, and also the expression of some inflammatory mediators.

Results: Irradiated fibroblasts have increased the invasiveness of the TNBC cell lines only, a stimulation that was prevented by CQ. On the other hand, invasiveness of the non-TNBC cell lines, which was not enhanced by irradiated fibroblasts, was also not significantly modified by CQ. In Balb/c mice, treatment with CQ prevented the stimulation of D2A1 TNBC cell migration in the pre-irradiated mammary gland, and reduced the number of circulating tumor cells and lung metastases. This protective effect of CQ was associated with a reduced expression of the inflammatory mediators interleukin-1β, interleukin-6, and cyclooxygenase-2, while the levels of matrix metalloproteinases-2 and -9 were not modified. CQ also promoted a blocking of autophagy.

Conclusion: CQ prevented radiation-enhancement of TNBC cell invasion and reduced the number of lung metastases in a mouse model.

Keywords: Chloroquine, inflammation, invasion, metastasis, radiation, triple negative breast cancer.

Background

Breast cancer is a heterogeneous disease, encompassing a number of distinct biological entities that are associated with specific morphological features and clinical behaviors.

Triple negative breast cancer (TNBC) accounts for 10–20% of all breast carcinomas and is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) [1]. Recurrence within 3 years of initial treatment is more likely for this aggressive form of breast cancer and results in a mortality risk two times higher than for non-TNBC patients [2]. Without any targeted therapies for TNBC, a better understanding and optimization of adjuvant treatment as radiotherapy remains essential.

Although radiotherapy is recommended to prevent locoregional relapse, the early recurrence found in some TNBC patients suggests that the formation of metastasis is favored in a subgroup of these patients who respond poorly to ionizing radiation. This stimulation of metastasis development could be related to the ability of radiotherapy to trigger an inflammatory response [3]. This inflammation is characterized by an increase of some cytokines and matrix metalloproteinases (MMP) that are known to favor metastasis development [4]. Further supporting this role of inflammatory cytokines, the association between a chronic inflammation and an increased risk of developing several types of cancer, including breast cancer, have been demonstrated [5]. But it is only recently that an acute inflammation induced by radiation in animal models has been associated with breast cancer progression [6, 7]. This feature of radiotherapy may be particularly important since radiation doses used in clinical practice do not always eradicate all cancer cells scattered in the breast. Such doses rather aim at optimizing long-term results with minimal adverse effects. It is therefore important to understand how an inflammation induced by radiation could accelerate the progression of breast cancer.

Enhancement of cancer cell invasion after their irradiation or exposure to free radicals has been reported for pancreatic cancer cells [8], as well as glioma [9], melanoma [10], colon carcinoma [11] and breast cancer cells [12]. These studies were designed to measure the invasiveness of irradiated cancer cells surviving radiation treatment. On the other hand, irradiating healthy tissues surrounding the tumor can also enhance cancer cell invasion. For instance, we showed that pre-irradiation of mouse mammary glands increased the migration of the mouse TNBC cell line D2A1, the number of circulating tumor cells, and favored the development of lung metastases [7]. Similarly, stimulation of cancer cell migration associated with inflammatory mediators has been reported after irradiation of a

mouse thigh and a rat brain [6, 13], demonstrating that certain inflammatory mediators stimulate the invasion of cancer cells which enter into the bloodstream and metastasize. These opposite effects of radiation, i.e. kill cancer cells or stimulate their invasiveness, could be particularly important for the TNBC subgroup that is at higher risk of early recurrence [14].

In the present study, we have determined whether administration of chloroquine (CQ) could prevent radiation-stimulated metastasis development in Balb/c mice. CQ is a large spectrum inhibitor used as antimalarial, anti-angiogenesis, autophagy inhibitor and anti-cancer drug [15]. It is also widely used as an anti-inflammatory agent for the treatment of rheumatoid arthritis and lupus erythematous [16, 17]. Because of the importance of inflammation in radiation-enhancement of breast cancer cell invasion, D2A1 mouse mammary carcinoma cell line was chosen instead of human xenografts tumors which require immunodeficient animals. The right third mammary gland of the mouse was irradiated prior the implantation of TNBC cells in order to better isolate the protective effect of CQ against radiation-induced inflammation in healthy tissue. Our study shows that CQ prevented the radiation-stimulated migration of D2A cancer cells in pre-irradiated mammary glands and reduced the development of lung metastases. As regular nonsteroidal anti-inflammatory drugs are usually prohibited during radiation therapy because of potential bleedings [18], CQ could be an interesting option as anti-inflammatory drug, to optimize the effects of this adjuvant treatment.

Methods

Cell culture

The TNBC cell lines D2A1, 4T1 and MDA-MB-231 and the non-TNBC cell lines MC7-L1, and MCF-7 were studied. The mouse breast carcinoma D2A1 cells, kindly provided by Dr. Ann F. Chambers (University of Western Ontario, London, ON, Canada), were derived from a spontaneous mammary tumor in a Balb/c mouse [19]. The mouse mammary carcinoma cell line MC7-L1 was generously provided by Dr Alfredo A. Molinolo of the Instituto de Biologia y Medicina Experimental, Concejo Nacional de Investigaciones Cientificas y Técnicas en Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina [20]. Other cell lines were purchases from American Type Culture

Collection (ATCC, Manassas, VA, USA). We confirmed the TNBC status of the D2A1 cells in collaboration with a pathologist of our institution pathology service using the clinical standard for immunohistochemistry protocols. Antibodies against ER and PR were used as well as Herceptest^{TM} for HER-2, all purchased from Dako (Burlington, ON, Canada). The receptor status for the 4T1, MDA-MB-231, MC7-L1 and MCF-7 cell lines were already reported (Table 1).

All cell lines were maintained in a 5% CO_2 humidified incubator at 37^{o}C in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC, Canada), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µM streptomycin.

Stable cell population of D2A1 encoding for the fluorescent ubiquitinated-based cell cycle indicator (FUCCI) proteins[33] were generated as previously described [7].

Table 1. TNBC status of the breast cancer cell lines

Cell lines	Species	Triple negative	References
MC7-L1	Mouse	No	[20]
4T1	Mouse	Yes	[44]
D2A1	Mouse	*Yes	Supplementary Figure S5
MCF-7	Human	No	[45, 46]
MDA-MB-231	Human	Yes	[46]

*TNBC status for the cell line D2A1 was determined as described in Materials and Methods

In vitro effect of CQ on cell growth and invasion capabilities

Effect of CQ on growth of the MC7-L1, 4T1, D2A1, MCF-7 and MDA-MB-231 cell lines was assessed. Cells (2.5×10^4) plated in 35 mm Petri dishes were either treated with medium (vehicle), 2.5 µM or 5 µM CQ, and their number was determined with a haemocytometer 24, 48 and 72 hrs later. The experiment was realized in triplicate and repeated 3 times.

For the invasion assay, conditioned media from irradiated Balb/c 3T3 fibroblasts were used as chemoattractant as previously described [7, 12]. Briefly, Balb/c 3T3 fibroblasts seeded in 24-well plates were irradiated using a ^{60}Co source (Gammacell 220, Nordion, Canada) at a dose of 5 Gy. Media were immediately removed after irradiation and replaced with DMEM supplemented with 0.1% BSA and CQ. Twenty-four hrs later, the conditioned media were isolated and used as chemoattractant in the lower compartment of invasion chambers (Becton Dickinson Biosciences, Bedford, MA, USA). Cancer cells were added to the upper compartment in DMEM 0.1% BSA supplemented with CQ. Cancer cells that crossed the layer of Matrigel™ were fixed 6 hrs (D2A1, 4T1) or 24 hrs later (MDA-MB-231, MCF-7, MC7-L1), stained with crystal violet and counted under the microscope. Results were reported as radiation-enhancement ratio. Each experiment was performed in triplicate and repeated two times.

Mammary gland pre-irradiation and implantation of D2A1 FUCCI cells

The experimental protocols were approved by the Université de Sherbrooke Ethics Committee for Animal Care and Use in accordance with guidelines established by the Canadian Council on Animal Care (Protocol ID number 013-14). An immunocompetent mouse model was preferred to human tumor xenografts implanted in nude mice in order to preserve the inflammatory response induced by radiation. Female retired breeder Balb/c mice (18 to 24 week-old) were obtained from Charles River (Raleigh, NA, USA). Animals were anesthetized with 3% isoflurane and then immobilized with a stereotactic mice frame adapted to dock on to a Leskell Gamma Knife® Perfexion™ (Elekta, Stockholm, Sweden). The third right mammary gland was irradiated daily with 4 fractions of 6 Gy (dose rate of 1.33 Gy/min) as previously described [7]. To determine whether pre-irradiation of the mammary gland stimulated the migration of mouse mammary cancer cells, D2A1 FUCCI-expressing cells (1×10^6/100 µl PBS) were implanted 3 h after the last irradiation into the

pre-irradiated (right side) and non-irradiated (control, left side) mammary glands. Mouse mammary carcinoma cells were also implanted into the mammary glands of sham-irradiated mice to analyze circulating tumor cells and lung metastases that were compared with pre-irradiated animals. Tumor volumes were measured every 3 days according to Balin-Gauthier et al. method [21]. Each experiment was performed in triplicate and repeated at least two times. In another group of animals, mice were euthanized to quantify pro-invasive molecules in mammary glands at different times post-irradiation.

CQ treatment

CQ purchased from Sigma-Aldrich (C6628, Oakville, Ontario, Canada) was injected intraperitoneally (i.p.) in Balb/c mice at 40 or 60 mg/kg suspended in 0.9% saline 3 h before each irradiation. Treatment was then administered every 72 hrs, which corresponds to the half-life of CQ, until euthanasia on day 21. Another group of mice were injected with saline 0.9% and used as non-treated control.

Quantification of circulating tumor cells

Blood samples were collected from the lateral saphenous vein of the sham and pre-irradiated mice, treated with vehicle or CQ at day 7 after the injection of D2A1 FUCCI-labeled cells into the mammary glands. Samples diluted 1:10 in PBS were spread in a Petri dish and covered with a glass cover slip. The presence of circulating tumor cells in each blood sample was quantified by fluorescence microscopy from 5 images of representative areas (magnification X 100). Fluorescence microscopy method was chosen instead of FACS analysis because repeated quantifications with small blood samples can be performed in the same animals.

***In vivo* and *in situ* optical imaging**

Migration of D2A1 FUCCI-expressing cancer cells in the mammary gland was monitored with an animal optical imager (QOS® Imager, Quidd S.A.S., Val de Reuil, France). Mice were anesthetized with ketamine/xylazine (87 : 13 mg/ml at 1 mg/kg). Bright field images of the mice were taken and then the appropriate filters were selected for red and green fluorescent image acquisition (mKO2, λ_{ex} = 472/30, λ_{em} = 536/40; mAG, λ_{ex} = 531/40, λ_{em} =

593/40). The three images were merged for future analysis. Distances of D2A1 cell migration in irradiated and non-irradiated mammary glands were measured to determine the radiation-enhancement ratio, and the protective effect of CQ. Migration was quantified with ImageJ (NIH, USA) as the distance from the nipple (physical landmark for the injection site) to the end of fluorescent smear. Animals were sacrificed on day 21 and tumor and lung specimens were removed. Fluorescence images of the lungs were acquired and the number of metastases was quantified. The diameter of the metastases was also measure using ImageJ. All quantifications were done for sham and irradiated mice, treated with vehicle, 40 mg/kg or 60 mg/kg CQ. Results are from 2 to 3 independent experiments, each realized in triplicate.

Histology

Mammary tumors and lung specimens containing D2A1 FUCCI-expressing cancer cells were collected and immediately frozen in a solution of Optimum Cutting Temperature (OCT; Electron Microscopy Sciences, Hatfield, PA, USA) or fixed with 4% paraformaldehyde for pathological examination using H&E staining by the Histology, Electron Microscopy and Phenotyping Services of Université de Sherbrooke. Invasion ratios were quantified on H&E staining using Nanozoomer Digital Pathology software. Cryosections of 3 or 7 µm were made using a Leica CM3050 Microsystems cryostat (Leica Microsystems Inc., Concord, ON, Canada). Slides were dried for 30 min at 37°C and then stored at -80°C until further use. The fluorescence emitted by the D2A1 cells was recorded using a FSX100® Bio Imaging Navigator microscope (Olympus, Center Valley, PA, USA) equipped with band pass filters (Chroma Technology Corp, Bellows Falls, VT, USA) for fluorescein isothiocyanate (FITC; λ_{ex} = 480/30, λ_{em} = 535/40) or tetramethylrhodamine isothiocyanate (TRITC; λ_{ex} = 560/40, λ_{em} = 630/60). To calculate the ratio of red and green fluorescence intensity of tumors cells, the entire slide was scanned (magnification X 42) and every image was quantified for red and green signals.

Immunohistochemistry

Immunohistochemistry assays were performed on tumor frozen sections (7 µm) to detect the CD31 blood vessel marker (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). An anti-goat secondary antibody conjugated with horseradish peroxidase was used for revelation (dilution 1:3000; Cedarlane, Burlington, ON, Canada) combined with the Dako EnVision HRP system (Burlington, ON, Canada). Tissues were counterstained with methyl-green. For each tissue, images of 10 representative areas were taken (magnification X 200) for signal quantification. The number of stained pixels were quantified using Pham *et al.* method [22] adapted by the Plateforme d'Analyse et de Visualization d'Images (PAVI) of the Université de Sherbrooke. The CD31 area (%) was calculated as the sum of CD31 stained pixels on the total pixels of each image X 100 and reported as radiation-enhancement ratios. Apoptosis in frozen tumor sections (3 µm) was quantified with an ApopTag® peroxidase *in situ* apoptosis detection kit (EMD Millipore, MA, USA) according to manufacturer's instruction. The percentage of positive cells was quantified in 10 representative areas (magnification X 200) for each tumor section. The results were reported as percentage of apoptotic cells.

Cell proliferation was measured by Ki67 marker in tumor paraffin-embedded sections. Tissues were deparaffinized with 3 consecutive baths of xylene and dehydrated with ETOH 95% and 70%. Tissues were boiled 3 min in citrate buffer pH 6.0 using a pressure cooker. Slides were incubated overnight at 4°C in a humid chamber with primary antibody (1:100, ab15580, Abcam, Toronto, ON, Canada) and then for 1 hour at room temperature with secondary antibody (1:1000, LS-C181152, LifeSpan BioSciences, Seattle, WA, USA). Tissues were counterstained with methyl-green, washed with xylene and sealed with Cytoseal^{TM} 60 mounting medium (18006, Electron Microscopy Sciences, Hatfield, PA, USA). The percentage of positive cells was quantified in 10 representative areas (magnification X 200) for each tumor section using Image-based Tool for Counting Nuclei plugin in imageJ software. The results were reported as percentage of positive cells.

Quantification of inflammatory and pro-migratory factors

The mRNA levels of cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and cytosolic phospholipase A2 (cPLA2) were determined by quantitative real-time

polymerase chain reaction (qPCR) in irradiated and contralateral non-irradiated mammary glands (n = 3) 6 hrs after the last session of irradiation as previously described [7].

Tissues were homogenized in 150 mM NaCl, 50 mM Tris pH 7.5, 1% triton, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. MMP-2 and MMP-9 were quantified by zymography, as previously described[6]. Autophagy markers LC3B1, LC3B2 and p62 were quantified by Western blot. Proteins were resolved in 15% acrylamide gel and transferred to PVDF membrane, which were probed with LC3B1+LC3B2 primary antibody (1:10 000, PA5-32254, Thermo Scientific, Rockford, IL, USA), p62 (1:1000, ab56416, Abcam, Toronto, ON, Canada) and secondary antibody (1:10 000 , LS-C181152, LifeSpan BioSciences, Seattle, WA, USA). The proteins were revealed by ECL Plus detection kit (PerkinElmer, Waltham, MA, USA). Relative intensity of the bands were normalized to beta-actin internal standard using ImageJ Gel Analyze function.

Statistical analysis

Experimental data are shown as mean ± standard error mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA) with multiple comparisons test. A P value of less than 0.05 was considered to be statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$.

Results

Radiation-stimulated invasion in TNBC cells was blocked by CQ

The ability of irradiated fibroblasts to increase the invasion of cancer cells was assessed in the TNBC cell lines D2A1, 4T1 (mouse) and MDA-MB-231 (human) and in the non-TNBC cell lines MC7-L1 (mouse) and MCF-7 (human). Used as chemoattractant, conditioned media from irradiated (5 Gy) 3T3 fibroblasts increased the invasiveness of all TNBC cell lines: D2A1; 1.7-fold ($****P < 0.0001$), 4T1; 1.8-fold ($***P < 0.001$) and MDA-MB-231; 5.8-fold ($****P < 0.0001$), compared to non-irradiated controls. On the other hand, no increase was measured with the non-TNBC cell lines MC7-L1 and MCF-7 (Figure 1A).

The ability of CQ to prevent this adverse effect of radiation was then assessed; but first, the concentration of CQ that does not modify the growth of these cancer cells was determined. Breast cancer cells were incubated with vehicle, 2.5 or 5 μM CQ and then counted 24, 48 and 72 hrs later (Figure 1B). CQ did not significantly decrease the cell proliferation, except for the 4T1 cell line for which a slower growth was measured for CQ but only after 72 hrs of incubation (CQ 2.5 μM; $****P < 0.0001$, CQ 5 μM; $****P < 0.0001$). This late effect was not a constraint since the invasion assays were completed in 6 hrs for this cell line. A concentration of 5 μM of CQ was therefore chosen.

For all the TNBC cell lines, treatment with CQ completely blocked the stimulation of their invasion induced by radiation (Figure 1A). It is noteworthy that CQ did not significantly reduce their basal invasion level measured without radiation. On the other hand, invasiveness of the non-TNBC cell lines MCF-7 and MC7-L1, which was not enhanced by irradiated fibroblasts, was also not significantly modified by CQ.

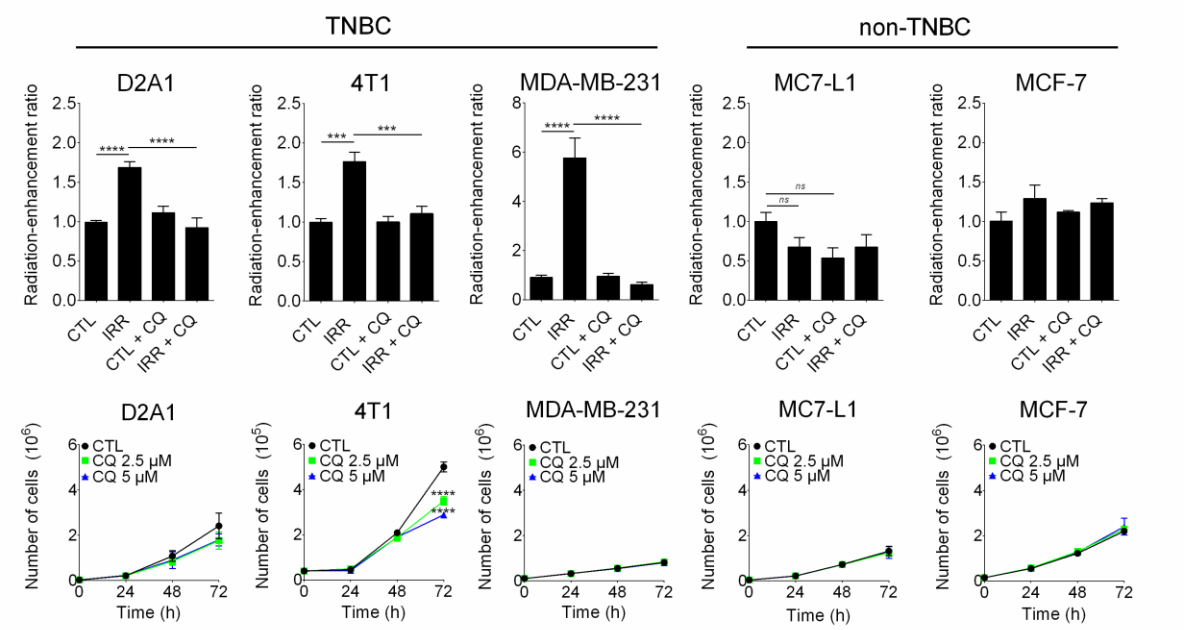

Figure 1. Effect of CQ on breast cancer cell invasion and growth. (A) Conditioned media from irradiated 3T3 fibroblasts was added in the lower compartment of invasion chamber and used as chemoattractant for breast cancer cells added in the upper compartment. Treatment with 5 µM CQ completely blocked radiation-enhancement of invasion in TNBC cell lines. Invasiveness of the non-TBNC cell lines were not modified by the irradiated 3T3 fibroblasts. CTL; Control, IRR; Irradiated, CQ; Chloroquine **(B)** Effect of CQ at 0, 2.5 or 5 µM on breast cancer cell growth measured 24, 48 and 72 h post treatment. Error bars indicate SEM. The experiment was realized in triplicate and repeated 3 times

Inhibition of D2A1 TNBC cell migration in mouse mammary gland

As previously reported, D2A1 tumors implanted in pre-irradiated mammary glands were significantly smaller compared to those in sham-irradiated mammary glands [7]. Treatment with CQ at 40 mg/kg before each session of irradiation, and thereafter at every 72 hrs, did not further affect tumor growth. The dose of CQ had to be increased to 60 mg/kg to measure a reduction in tumor volume that was significant from day 18 in non-irradiated animals, and from day 21 in tumors implanted in pre-irradiated mammary glands (Figure 2A). To exclude systemic effect of radiation on tumor growth, tumor volumes of sham-irradiated animals (sham tumors) were compared to control tumors (left side) of pre-irradiated animals as a validation of the mice as its own control in following experiments (Figure S1).

The effect of CQ on radiation-stimulated migration of D2A1 cells was then assessed. As measured with an animal optical imager, pre-irradiation of the mouse mammary gland increased by 1.7-fold (**P < 0.01) the distance of D2A1 cell migration. This stimulation was completely prevented by treating the animals with CQ at 40 mg/kg (*P < 0.05) or 60 mg/kg (**P < 0.01) (Figure 2B and C). These results were then confirmed by H&E staining (Figure 2D and E).

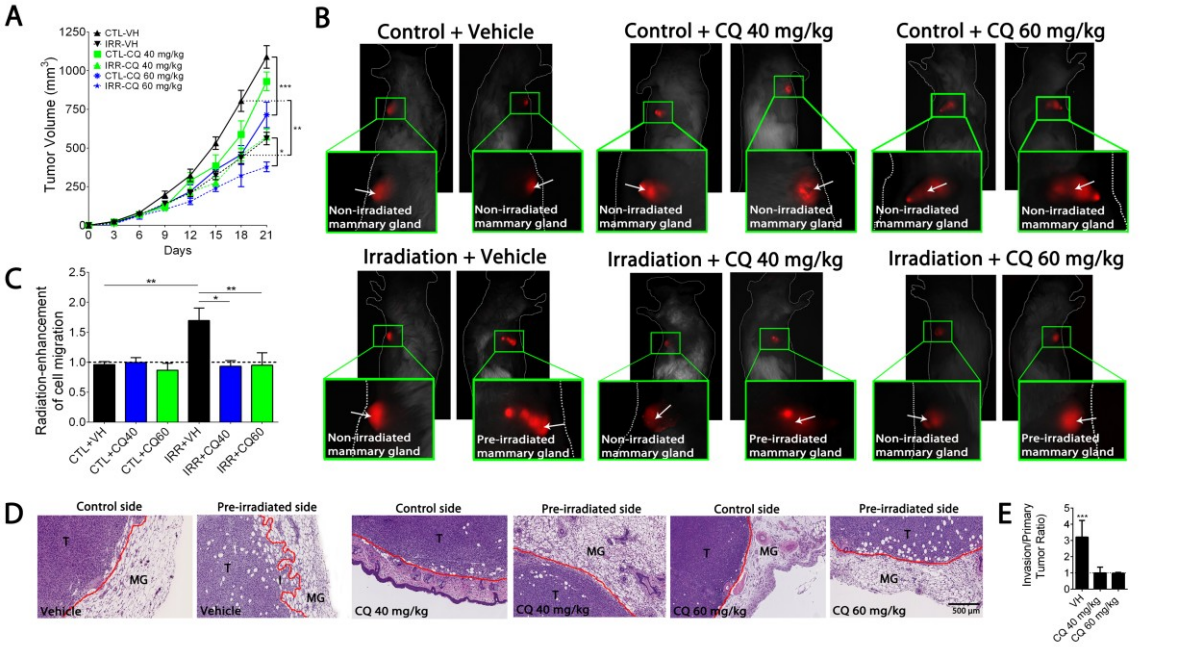

Figure 2. Effect of CQ on D2A1 tumor growth and migration. (A) D2A1 tumor volumes measured after implantation in pre-irradiated or non-irradiated mammary glands of animals treated with vehicle or CQ. Treatment with CQ at 60 mg/kg significantly reduced the tumor volume from day 18 in non-irradiated animals, and from day 21 in tumors implanted in pre-irradiated mammary glands. **(B)** and **(C)** *in vivo* optical imaging of D2A1 cells in mice mammary glands. White arrows = injection site of D2A1 cells. Cell migration in pre-irradiated mammary glands was enhanced by 1.7-fold (**P < 0.01) compared to control side. Treatment with CQ at 40 mg/kg (*P < 0.05) or 60 mg/kg (**P < 0.01) completely blocked radiation-stimulation of cell migration in mammary glands. **(D)** H&E staining from tumor sections confirming results observed in B and C. T = D2A1 tumor, MG = mammary gland. **(E)** Quantification of tumor invasion using H&E staining. Invasion was calculated as follow: Invasion area (mm^2)/Primary tumor area (mm^2). Results were reported as radiation-enhancement ratio. H&E quantification of tumor sections show a 3.2-fold increase of invasion (***P = 0.004) for tumors implanted in pre-irradiated mammary glands that was completely prevented using CQ.

Reduction of tumor vascularization

Since the anti-angiogenic ability of CQ was previously reported [16], we determined whether this effect of CQ was associated with the inhibition of radiation-enhancement of TNBC cell migration. Pre-irradiation of the mammary gland before implantation of D2A1 tumors did not modify the tumor vascularization compared to tumors implanted in non-irradiated mammary glands, as measured with blood vessel marker CD31. On the other hand, CQ treatment significantly decreased the level of CD31 in tumors implanted in the pre-irradiated and non-irradiated mammary glands (Figure 3). This reduction was similar for the two doses of CQ studied.

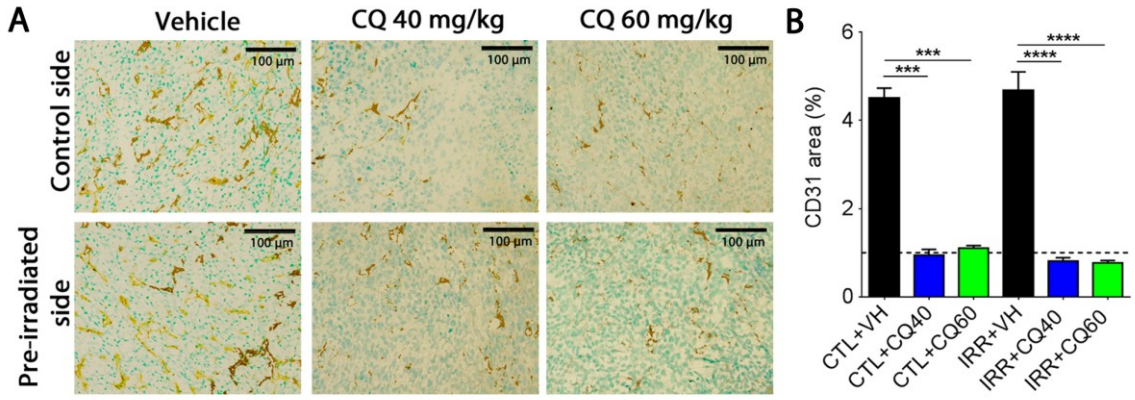

Figure 3. Effect of CQ on tumor vascularization. (A) Immunohistochemistry against CD31 endothelial marker in frozen tumor sections (magnification X 200). **(B)** Quantification of CD31 signal plotted as percentage of stained area between control (sham) vs control + CQ, or irradiated vs irradiated + CQ. ***$P < 0.001$, ****$P < 0.0001$. Error bars indicate SEM for n = 3 to 14 independent experiments for each group.

Effect on cell cycle distribution

In our model, the FUCCI colorimetric vectors expressed by the D2A1 cells generate a green fluorescence when cells are in the $S/G_2/M$ phases and red fluorescence for the G_1/G_O phases. Using these fluorescent makers, distribution of $S/G_2/M$ and G_1/G_O phases was determined in frozen sections of tumors implanted in control or pre-irradiated mammary glands. Stimulation of cancer cell migration in pre-irradiated mammary gland was associated with an enrichment of D2A1 cells in G_1/G_O phases (red fluorescence) by 36.4% and a decrease in $S/G_2/M$ phases (green fluorescence) by 11.7%. Treatment with CQ has completely prevented this enrichment in the G_1/G_O phases, as well as the decrease of cells in $S/G_2/M$ (Figure 4A and B).

The cell proliferation marker Ki67 was then used to further assess the effect of radiation and CQ on D2A1 cell proliferation. Treatment with CQ at 40 and 60 mg/kg increased by 2-fold the levels of Ki67 expressed in D2A1 tumors (Figure 4C). Since the Ki67 marker is absent from cells in G0 phase, this suggests that CQ has induced a transfer from quiescent to cycling cell state. Control tumors were also compared with sham tumors to exclude possible radiation-induced systemic bias on proliferation (Figure S2).

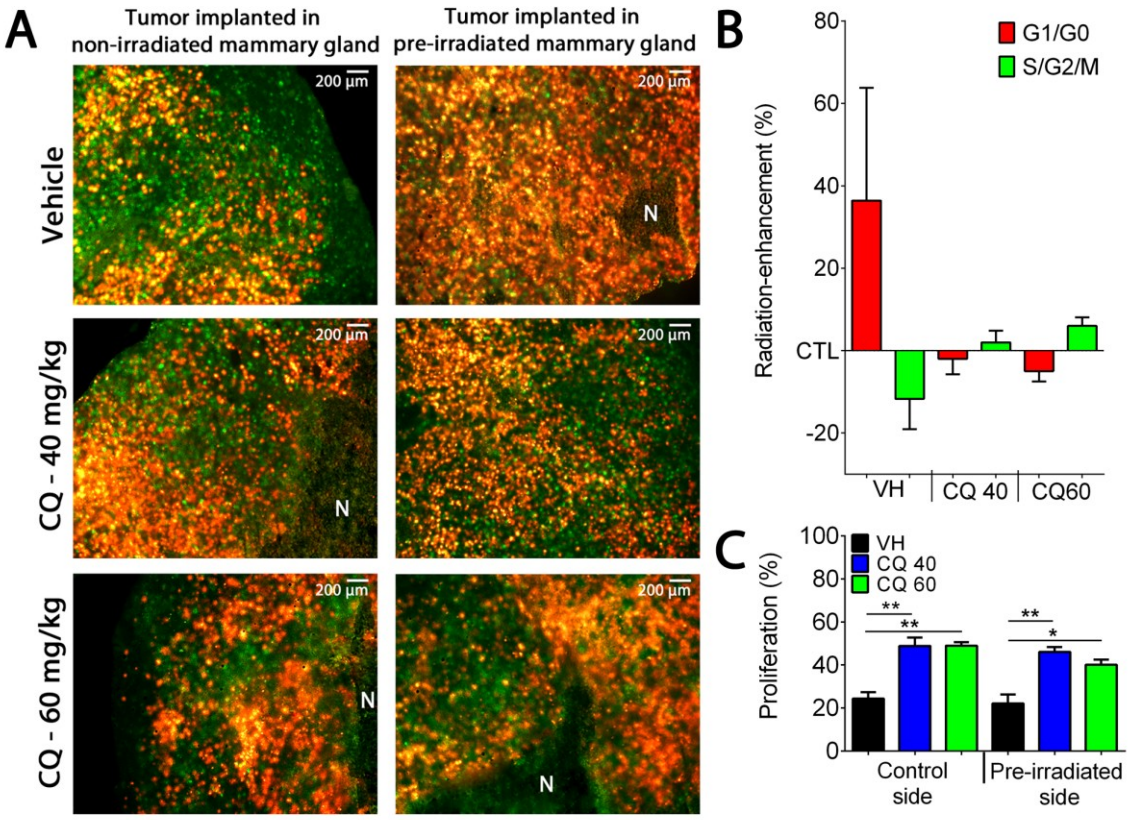

Figure 4. Effect of CQ on cell cycle distribution in D2A1 FUCCI tumors. (A) Representative fluorescence images of frozen sections of mammary tumors used to quantify cancer cells in S/G2/M (green) or G1/G0 (red) phases. **(B)** Effect of radiation on cell cycle distribution plotted as radiation-enhancement ratio of red and green cells in percentage. **(C)** Quantification of Ki67 by immunohistochemistry on D2A1 tumor frozen sections. $*P < 0.05$, $**P < 0.01$. Error bars indicate SEM for n = 4 to 11 independent experiments for each group.

Reduction of lung metastasis development induced by radiation

The preventive effect of CQ on the development of lung metastasis stimulated by radiation was first assessed by quantifying the number of circulating tumor cells (CTC). In the first group of mice, the right mammary gland was pre-irradiated before implantation of D2A1 cells on both sides, while in the second group, the D2A1 cells were also implanted in both mammary glands but in sham-irradiated animals. As we previously reported, pre-irradiation of the mammary gland before the implantation of D2A1 cells increased the number of CTC as well as the number of lungs metastases by 2.4-fold compared to sham-irradiated mice [7]. CQ treatment with 40 mg/kg and 60 mg/kg completely prevented the radiation-enhancement of CTC which came back to the basal level found in sham-irradiated animals

(Figure 5A). Consequently, CQ also prevented the development of lung metastasis induced by radiation (Figure 5B and C), but did not affect their diameter (Figure 5D). Interestingly, CQ did not decrease the basal number of lung metastases compared to sham-irradiated animals that received the vehicle. These results suggest that CQ selectively targeted a pathway associated with the radiation-stimulated development of lung metastasis.

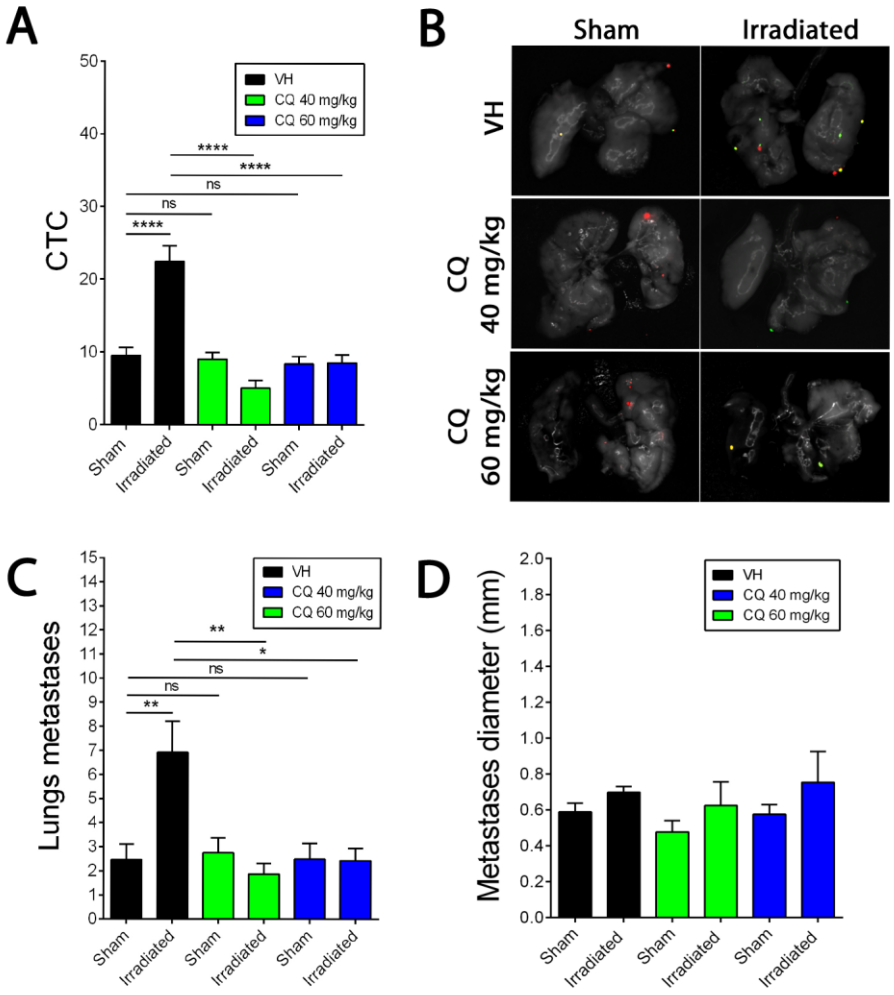

Figure 5. Inhibition of radiation-enhancement of lung metastases with chloroquine. **(A)** Quantification of circulating tumor cells in blood samples of sham and irradiated mice. **(B)** Optical imaging of lung metastases. ****$P < 0.0001$. **(C)** Quantification of the number of lung metastases. *$P < 0.05$, **$P < 0.01$. Sham: Non-irradiated animals with tumor implantation on both sides. Irradiation: Pre-irradiation of the right mammary gland following by tumors implantation on both sides. **(D)** Quantification of the diameter of lung metastases from optical imaging results. No significant difference was observed for sham or irradiated mice, as for chloroquine treatment. Error bars indicate standard error of the mean (SEM) for n = 4 to 15 animals for each group.

Effect of CQ on apoptosis and autophagy in D2A1 tumors

To further assess how CQ prevented the formation of new metastases, apoptosis and autophagy were measured in D2A1 tumors. Treatment with 40 mg/kg of CQ did not significantly modify the percentage of apoptotic cells. An increase by 3-fold compared to vehicle was observed at 60 mg/kg CQ, but only in tumors implanted in pre-irradiated mammary glands (****$P < 0.0001$) (Figure 6A).

Quantification of autophagy markers LC3B1 and 2 by Western blot was then performed in tumor homogenates. As expected, the expression of LC3B2 was increased by radiation, supporting an accumulation of autophagosomes. This accumulation was then confirmed to be an increase of autophagy since there is no accumulation of the p62 marker. On the other hand, the blockage of autophagy, preferentially in tumors implanted in pre-irradiated mammary glands, was supported by the accumulation of p62 in CQ-treated tumors, which is usually degraded when autophagy is activated (Figure 6B and S3). Radiation-induced systemic bias on autophagy were excluded by comparing autophagy marker in sham and control tumors (Figure S3 and S4). Overall, autophagy was preferentially induced in tumors implanted in pre-irradiated mammary glands underlying the importance of tumor microenvironment affecting the tumor.

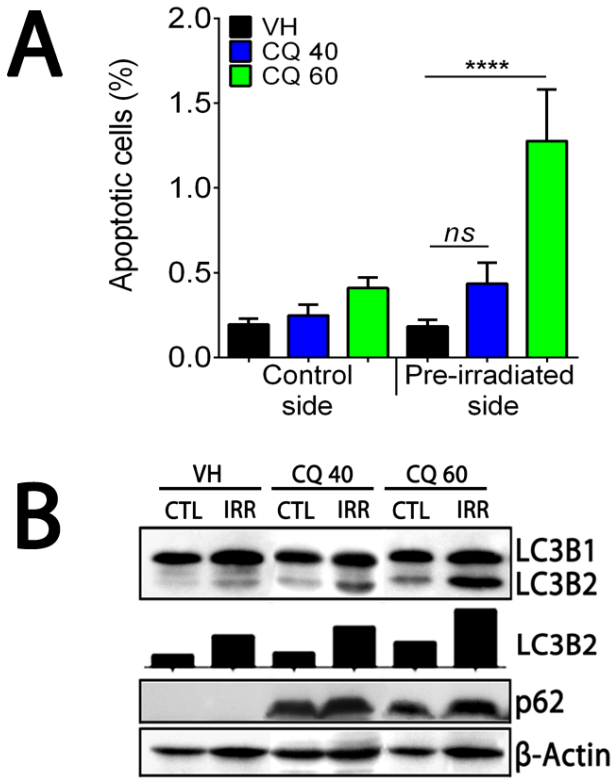

Figure 6. Apoptosis and autophagy analyses of D2A1 tumors (A) TUNEL assay quantification of the percentage of apoptotic cells in tumor sections of each groups of mice. ****$P < 0.0001$. Error bars indicate SEM for n = 3 to 6 independent experiments. **(B)** Immunoblot of protein lysates from D2A1 tumors for autophagy markers. Experiment was realized in triplicate.

Assessment of pro-migratory and inflammatory factors

To characterize these adverse effects of radiation, some pro-migratory and inflammatory factors were quantified in pre-irradiated and control mammary glands. A CQ dose of 40 mg/kg was chosen to exclude the induction of cell death occurring at higher doses.

The proteases MMP-2 and MMP-9 are known to favor the migration and invasion of cancer cells. Their levels were determined by zymography in mammary glands 6 hrs after the last irradiation and 21 days after D2A1 tumor implantation (Figure 7A and B). Radiation did not increase the levels of MMP-2 and -9 in the mammary glands that were implanted/not implanted with the D2A1 tumor. The level of either of these proteases was not reduced after treatment with CQ at 40 mg/kg.

Expression of some inflammatory mediators potentially involved in cancer cell invasion were then quantified (Figure 7C). The relative mRNA levels of IL-1β and IL-6 were significantly increased 6 hrs post-irradiation, as measured by qPCR. Regarding the pathway of prostaglandins, a higher expression of COX-2 and cPLA2 were also measured in irradiated mammary glands. Treatment with CQ significantly decreased the expression of IL-1β and IL-6 in both irradiated and non-irradiated mammary glands, and completely inhibited the stimulation COX-2 and cPLA2 induced by radiation.

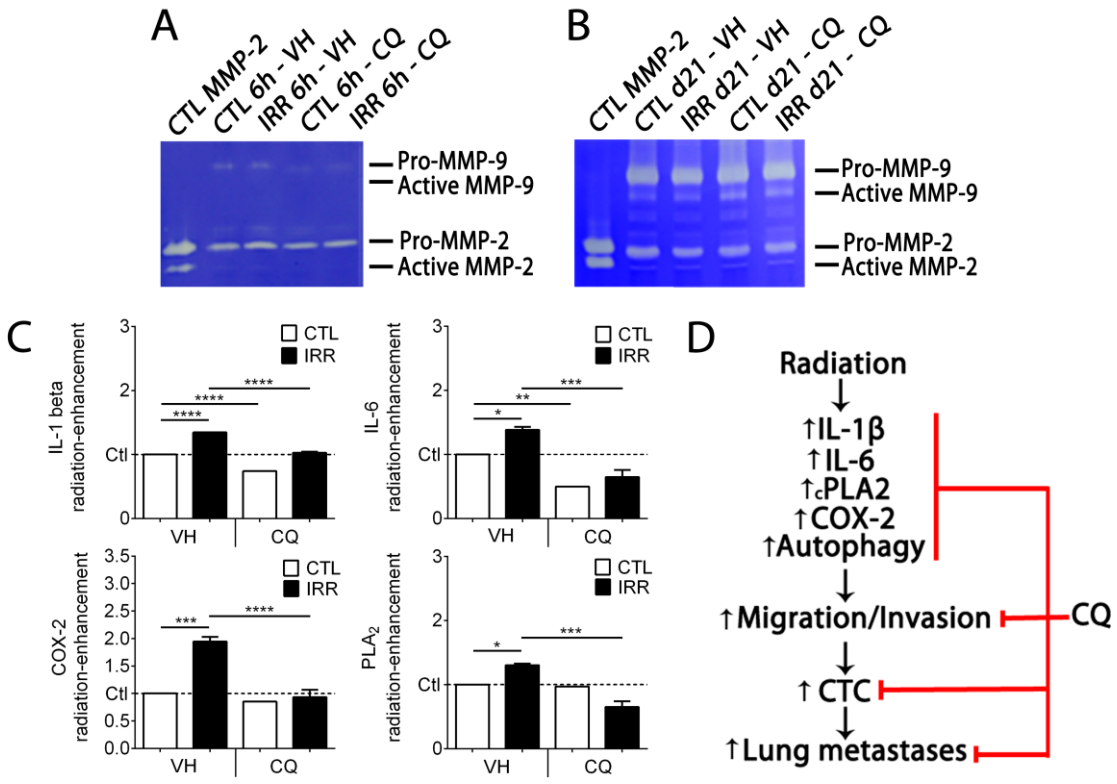

Figure 7. Quantification of pro-migratory and pro-inflammatory factors after chloroquine treatment. (A) Zymogram of MMP-2 and -9 levels after chloroquine treatment performed on protein lysates of both irradiated and non-irradiated mammary glands collected 6 h after irradiation. **(B)** Zymogram of MMP-2 and -9 levels after chloroquine treatment performed on protein lysates of D2A1 tumors implanted in pre-irradiated and non-irradiated mammary glands collected on sacrifice day (day 21). **(C)** Effect of chloroquine 40 mg/kg on the relative expression of pro-inflammatory genes potentially in mammary quantified by qPCR 6 h after the last session of irradiation. Relative mRNA expressions are plotted as a radiation enhancement ratio. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. **(D) S**ummary of the proposed mechanism of chloroquine in the prevention of TNBC invasion stimulated by radiotherapy. Error bars indicate SEM. Experiments were realized in triplicate.

Discussion

For the subgroup of TNBC patients that responds poorly to radiotherapy, the risk of recurrence is very high during the first three years after treatment and cure is unlikely [23]. The concept of radiation-stimulated cancer cell migration and invasion is well accepted [24], but the hypothesis suggesting that formation of metastasis could be stimulated by radiation in some TNBC patients still need to be validated. Meanwhile, it has been shown in our previous pre-clinical study that pre-irradiation of a Balb/c mouse mammary gland increased the migration of murine TNBC cells, the number of CTC and favored the development of lung metastases [7]. By irradiating the mammary gland prior to implantation of TNBC cells, this previous study properly demonstrated the contribution of inflammatory mediators released from healthy tissues on metastasis development.

In the present study, we first showed that these adverse effects of radiation were observed *in vitro* only in the TNBC cell lines and that they can be prevented by CQ. It should be noted that fibroblasts were used to mimic the stroma in invasion chambers but the role of other stromal components in radiation-enhancement of breast cancer cells should not be excluded and requires further investigation. Also, it remains to be determined why radiation did not stimulate the invasion of non-TNBC cancer cells. Also, it is noteworthy that the protective effect of CQ *in vitro* was not related to inhibition of cancer cell proliferation since no significant effect on cell growth was measured.

Accumulation of CQ in the trans-Golgi network leads to its alkalinization which deregulates the maturation of many proteins, including MMP. MMP-2 and -9 play an important role in cancer cell migration and invasion by cleaving proteins of the extracellular matrix [25, 26]. In the present study, no increase of MMP-2 and -9 was found in irradiated Balb/c mouse mammary gland, and treatment with CQ did not reduce their basal levels. However, a possible involvement of these MMP in breast cancer cell invasion cannot be ruled out since an increased activity of these MMP and a stimulation of cancer cell invasion was observed in other pre-clinical models such as irradiated mouse thigh and rat brain [6, 13]. In breast cancer patients, radiotherapy can increase the plasma level of MMP-9 [27] and the level of MMP-2 was also significantly higher in skin biopsies of women after radiotherapy, relative to non-irradiated skin [28]. On the other hand, reduction of MMP-2 and -9 expression *in vitro* in the MDA-MB-231 cells was reported at higher

doses of CQ than used in our study [29]. Therefore, it remains to be determined in TNBC patients whether radiation can increase the expression of MMP-2 and -9, and whether this can be prevented by CQ.

It was reported that the development of radiation-stimulated lung metastasis after the irradiation of the mammary gland was correlated with inflammatory pathways involving COX-2 as well as IL-1β and IL-6 cytokines [7]. As CQ is also used as an anti-inflammatory agent for the treatment of rheumatoid arthritis and lupus erythematous [16, 17], we determined whether its anti-cancer effect could be associated with a down-regulation of these inflammatory pathways.

In irradiated mouse mammary glands, the stimulation of $cPLA_2$ (the first enzyme in the production of prostaglandins) and COX-2 expression were completely prevented by CQ treatment. This inhibitory effect of CQ may have a major impact on breast cancer patient survival. Indeed, elevated expression of COX-2 was associated with poor prognosis and distant metastases in TNBC patients [30, 31], while radiation-enhancement of cancer cell invasion as assessed *in vitro* can be completely prevented by adding a COX-2 inhibitor [12]. These results support the hypothesis that the inhibition of COX-2 may increase the disease free-survival of TNBC patients, as previously observed for early stage non-TNBC patients [32].

It is noteworthy that CQ did not reduced the basal levels of cPLA2 and COX-2 measured in non-irradiated mammy glands. Since COX-2 is inducible only under pathological or inflammatory conditions, this may suggest that the effect of CQ would be specific to irradiated tissues, resulting in fewer adverse effects for non-irradiated healthy tissues.

We previously reported that the inflammatory cytokine IL-1β was increased in the conditioned media of fibroblasts following radiation. In the same study, IL-1β stimulated the invasiveness of MDA-MB-231 TNBC cells, and this invasive effect was prevented by adding an anti-IL-1β antibody [33]. The resulting enhancement of the invasion appears to be related to an increased expression of COX-2, since the addition of a COX-2 inhibitor completely prevented the stimulation of cancer cell invasion induced by IL-1β [12, 33]. In our mouse model of TNBC, the protective effect of CQ on metastasis development was

also associated with a reduction of IL-1β expression, suggesting that this cytokine is a primary target of CQ in the development of lungs metastases.

Regarding IL-6, it is the most important cytokine associated with poor prognosis for breast cancer, and it is known for controlling breast cancer cell growth and regulating cancer stem cell renewal [34]. IL-6 has also been reported to stimulate the proliferation and migration of breast cancer cells *in vitro* as well as tumor progression [35], but its potential connection with radiotherapy was less studied [34]. Nevertheless, Yu *et al*. reported that radiation-induced IL-6 in MDA-MB-231cells promoted the invasion and migration of non-irradiated neighboring cells [36]. In our mouse model, CQ reduced the expression of IL-6 in irradiated and non-irradiated mammary glands in the same manner observed with IL-1β suggesting that this cytokine could also be associated with induction of lung metastasis.

Irradiation of healthy tissues surrounding a tumor can modify the balance between proliferation and migration of cancer cells [7, 13]. This migration/proliferation dichotomy was described as mutually exclusive or as a «Go or Grow» phenomenon [37]. Using the FUCCI cell cycle reporter system [38], irradiation of a rat brain or a mouse mammary gland favored the migration of cancer cells and their accumulation in the G_1/G_0 phases [7, 13]. This suggests that cytokines released from irradiated tissues could stimulate the migration/invasion of cancer cells through a reduction of their proliferation. Treatment with CQ has successfully reduced the radiation-enhanced accumulation of D2A1 cells in the G_1/G_0 phases (red fluorescence), supporting the inhibition of radiation-induced migration in mammary glands. These results are consistent with the decrease of G_1/G_0 cells after CQ treatment previously observed in human TNBC cell lines by Jiang *et al.*[39]. The authors reported the induction of cell cycle arrest in G2/M which may affect the interpretation of cell proliferation with the marker Ki67. This marker of cell proliferation is present in both G2 and M phases. Consequently, an arrest in G2/M may increase the number of Ki67 positive cells, giving the false indication that more tumor cells are proliferating. Indeed, the increased number of Ki67 positive cells measured in our study is expected to be associated with a cell blockage in G2/M rather than an increase of cell proliferation.

The reduction of CTC and the number of lung metastases was not caused by a reduction of tumor blood supply since the presence of CD31 blood vessel marker was not

affected by radiation. It was then impossible to associate the protective effect of CQ with the reduction of tumor vascularization.

Our results showed that stimulation of metastasis development stimulated by radiation was inhibited by CQ without affecting the tumor volume. Our results also showed that a low level of apoptosis was only promoted in D2A1 tumors with high dose of CQ (60 mg/kg) in presence of radiation but not with 40 mg/kg of CQ. This suggests that the adverse effect of radiation on the development of metastasis can be prevented by low doses of CQ that would not induce apoptosis in healthy tissues. Consequently, a low systemic toxicity after treatment with CQ could be expected.

CQ is also described as an inhibitor of autophagy. Autophagy is a survival pathway activated in response to stress whereby cellular components are degraded to recycle energy, promote cell survival and cancer resistance. However, if the cells cannot recover from the damage, autophagy will ultimately lead to cell death. Therefore, autophagy could also exert a significant control over the progression of cancer and tissue homeostasis [40]. Our results showed that treatment with CQ blocked autophagy. These findings are consistent with those of Jensen *et al*.[41], who reported that CQ was highly effective in preventing autophagy. These authors also reported that CQ preferentially accumulated in acidic tumor environment than in normal tissue, suggesting that CQ could be less non-toxic for normal tissues. The increase of autophagy observed in tumor implanted in pre-irradiated tissue could be directly associated to this previous observation. Overall, according to the experimental conditions, autophagy can be either cytotoxic (prolonged autophagy will eventually lead to cell death) or cytoprotective (survival mechanism for the cell). Autophagy is clearly a complex process and its role in TNBC patients remains to be further explored. Without knowing how exactly autophagy was regulated, the preferential blocking of autophagy associated with the accumulation of LC3B2 observed for tumors implanted in pre-irradiated mammary glands seems to be associated with the prevention of the radiation-stimulated of breast cancer cell migration.

Combined with radiation, CQ successfully induced cell death in several human TNBC cell lines [42, 43]. Zhao *et al.* have shown the radiosensitivity potential of CQ in MDA-MB-231 TNBC cells, by reporting enhanced apoptosis and necrosis [42]. In our study, the mammary gland was irradiated before its implantation with D2A1 cells.

Therefore, the anti-cancer effect of CQ cannot be related to a direct radiosensitization but rather to an indirect effect on cancer cells that is mediated by irradiated stroma. The experimental protocol used in this study has provided to confirm that CQ prevents the stimulation of the metastasis development induced by the irradiated stroma. Taken together, these results suggest that treating TNBC patients with CQ could further increase the anti-tumor effect of radiotherapy and reduce the potential adverse effects of radiation-induced inflammation on the stimulation of metastasis development.

Conclusion

In conclusion, the ability of radiation to stimulate the invasion of cancer cells was observed *in vitro* only in TNBC cell lines. In our mouse model of TNBC, radiation stimulates the cancer cell migration and development of metastasis which seems to involve multiple inflammatory pathways including those of COX-2, IL-1β and IL-6. These adverse effects of radiation were prevented by treating the animals with CQ. A proposed mechanism is presented in Figure 7D. Based on these results, a clinical trial to determine whether treatment with CQ could increase the disease-free survival of the TNBC patients that poorly respond to radiation treatment could be undertaken.

Declarations

List of abbreviations

BSA: Bovine serum albumin; CD31: Cluster of differentiation 31; cPLA2: cytosolic Phospholipase A2; Co: Cobalt; COX-2: Cyclooxygenase-2; CQ; Chloroquine; CTC: Circulating tumor cell; CTL: Control; Dulbecco modified Eagle's medium: DMEM, ER: Estrogen receptor; FITC: Fluorescein isothiocyanate FUCCI: Fluorescent ubiquitinated-based cell cycle indicator; H&E: Haematoxylin and eosin; HER2; Human epidermal growth factor receptor 2; HRP: Horse radish peroxidase; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; i.p.: intraperitoneal; IRR: Irradiated, LC3: Light chain 3; mAG: **monomeric Azami Green;** mKO2: **monomeric Kusabira Orange 2;** MMP: Matrix metalloproteinase; OCT: Optimum cutting temperature; PR: Progesterone receptor; qPCR: quantitative Polymerase chain reaction; TNBC: Triple negative breast cancer; TRITC: Tetramethylrhodamine isothiocyanate; VH; Vehicle

Ethics approval

The experimental protocols were approved by the Ethics Committee for Animal Care and Use of the Université de Sherbrooke in accordance with guidelines established by the Canadian Council on Animal Care (Protocol ID number 013-14).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Authors' contributions

GB performed all animal experiments, analyses, results interpretation and drafted the manuscript. HT contributed to *in vitro* experiments and generated FUCCI cells. GB, CS and BP conceptualized the study. SG contributed to pathological analysis. YBL contributed to *in vivo* imaging experiments. BP, CS, RB, YBL and SG contributed to writing and revising the manuscript. All authors contributed to critical analysis and approval of the final manuscript.

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End notes

Not applicable.

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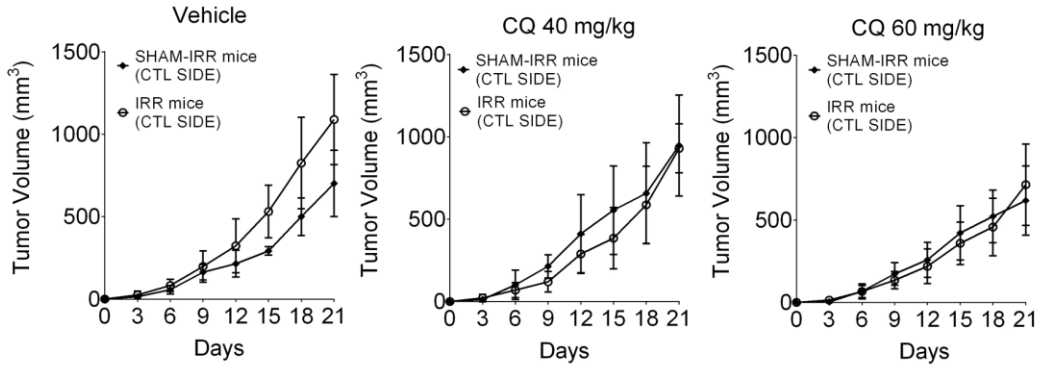
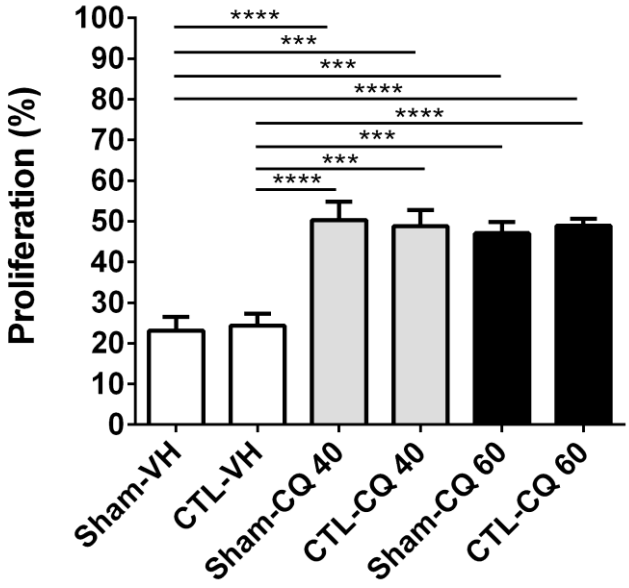
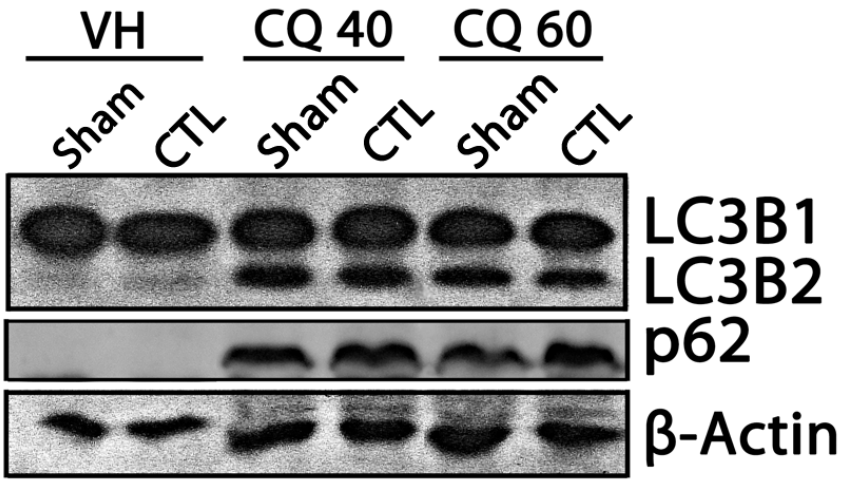
Supplementary material



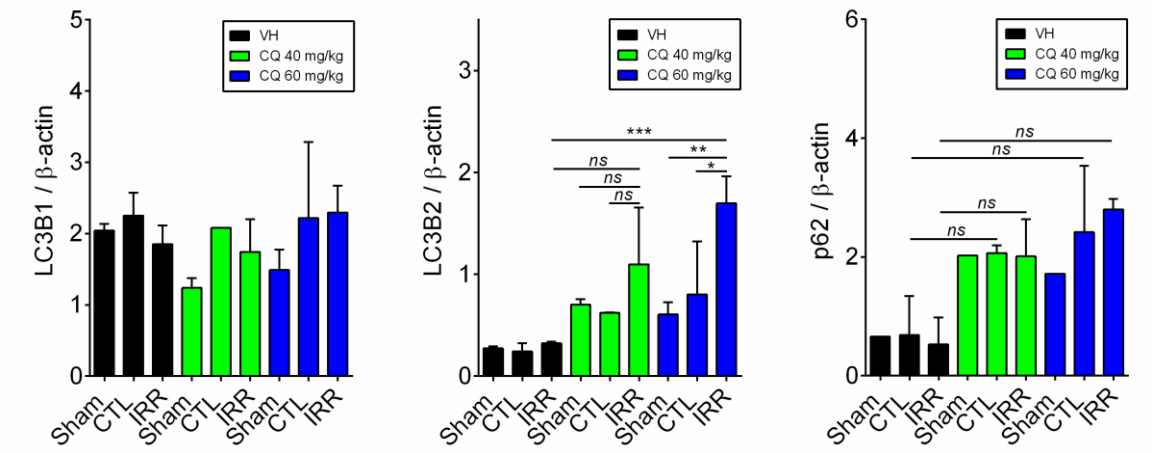
Supplementary figure S1. Validation of the mice as its own control in mice pre-irradiated at the right mammary gland. D2A1 tumor volumes of sham irradiated animals (sham tumors) were compared to control tumors (left side) of pre-irradiated animals. Error bars indicate s.e.m. for n = 6 to 15 animals for each group.



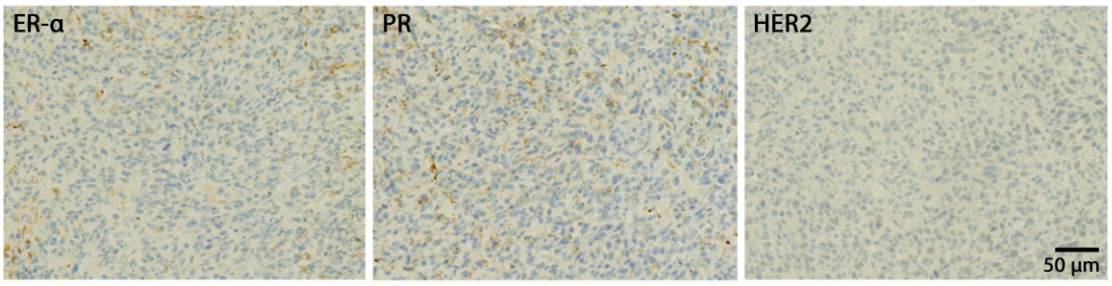
Supplementary figure S2. Ki67 immunohistochemistry in sham (non-irradiated animals) and control tumors (left side of pre-irradiated animals) were realized to exclude possible systemic effect of radiation on tumor proliferation. The experiment was realized in triplicate. Sham-VH vs Sham-CQ 40; $P < 0.0001$, Sham-VH vs CTL-CQ 40; $P = 0.0002$, Sham-VH vs Sham-CQ 60; $P = 0{,}0001$, Sham-VH vs CTL-CQ 60; $P < 0{,}0001$, CTL-VH vs Sham-CQ 40; $P < 0{,}0001$, CTL-VH vs CTL-CQ 40; $P = 0{,}0002$, CTL-VH vs Sham-CQ 60; $P = 0{,}0001$, CTL-VH vs CTL-CQ 60; $P < 0{,}0001$.



Supplementary figure S3. Immunoblot of autophagy markers were realized in sham (non-irradiated animals) and control tumors (left side of pre-irradiated animals) to exclude possible systemic effect of radiation on tumor autophagy. The experiment was realized in triplicate.



Supplementary figure S4. Quantitative densitometry from Western blots of the expression of **(A)** LCB3I, **(B)** LCB3II (Sham-CQ 60 vs IRR-CQ 60; $P = 0.0024$, CTL-CQ 60 vs IRR-CQ 60; $P = 0.0182$, IRR-VH vs IRR-CQ 60; $P = 0.0009$) and **(C)** p62 autophagy markers calculated using ImageJ Gel Analyze function.



Supplementary figure S5. Hormonal status of D2A1 cell line was confirmed by immunohistochemistry as described in Materials and Methods. No nuclear (ER and PR) as well as membrane (HER2) staining were observed. D2A1 cells were then revealed to be triple negative by a pathologist of our institution.

OBJECTIF #2

Article 4: Radiation-induced lung metastasis development is MT1-MMP-dependent in a triple negative breast cancer mouse model

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Statut de l'article: Soumis

Référence: Bouchard G, Therriault H, Sameh Geha, Bujold R, Saucier C and Paquette B (2016) Radiation-induced lung metastasis development is MT1-MMP-dependent in a triple negative breast cancer mouse model. *British Journal of Cancer* (submitted).

Avant-propos: J'ai participé à la conception de l'étude avec Pr Benoit Paquette. J'ai effectué toutes les expériences *in vivo*, l'imagerie et l'analyse des tissus conséquents. Hélène Therriault a généré les lignées cellulaires réprimées pour la MT1-MMP ainsi que les chambres d'invasion. J'ai analysé tous les résultats et j'ai rédigé la première ébauche du manuscrit. En collaboration avec les autres auteurs, j'ai participé à l'amélioration de l'article jusqu'à sa version finale.

Résumé: Pour un sous-groupe de patientes atteintes de TNBC, la récidive peut arriver très tôt pendant les trois premières années après le traitement et la guérison est peu probable en cas de récidive. Des études précédentes suggèrent que l'inflammation radio-induite pourrait promouvoir la progression du cancer, augmentant ainsi le risque de récidive précoce. En utilisant un modèle de souris TNBC, nous avons déterminé si l'expression de la MT1-MMP est associée au développement de métastases pulmonaires radio-induites. À partir des cellules de carcinome mammaire de souris D2A1, de nouvelles lignées exprimant 40% et 70% d'ARNm de la MT1-MMP ont été comparées avec les D2A1-wt (lignée originale; *wild type*) et les D2A1 shMT1-mock (vecteur vide). La pré-irradiation de la glande mammaire suivie de l'implantation des cellules D2A1-wt ou D2A1-mock a mené à une augmentation du nombre de CTC ainsi que des métastases pulmonaires. Cette progression radio-induite du cancer a été associée à une localisation nucléaire de la MT1-MMP, mais a été complètement inhibée en réprimant l'expression de cette même protéase. En conclusion, ce modèle pré-clinique suggère qu'un seuil minimal de MT1-MMP est requis pour stimuler l'invasion radio-induite et que son expression et/ou localisation pourraient être de bons biomarqueurs de prédiction de l'efficacité de la radiothérapie chez les femmes atteintes de TNBC.

Radiation-induced lung metastasis development is MT1-MMP-dependent in a triple negative breast cancer mouse model

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Abstract

Background: Using a mouse model of triple negative breast cancer (TNBC), we have determined whether expression of the cell membrane protease MT1-MMP in cancer cells was associated with radiation-stimulated development of lung metastases.

Methods: Irradiated fibroblasts were used as chemoattractant to assess in invasion chamber the invasiveness of TNBC D2A1 cell lines showing a downregulated expression of MT1-MMP, which were compared to D2A1-wt (wild-type) and D2A1 shMT1-mock (empty vector) cell lines. In a mouse model, a mammary gland was irradiated followed by the implantation of the downregulated MT1-MMP D2A1, D2A1-wt or D2A1 shMT1-mock cell lines. Invasion of D2A1 cells in the mammary gland, number of circulating tumor cells and development of lung metastases were assessed.

Results: Reduction of MT1-MMP expression decreased the invasiveness of D2A1 cells and blocked the radiation-enhancement of cancer cell invasion. In BALB/c mice, irradiation of mammary gland has stimulated the invasion of cancer cells, which was associated with a higher number of circulating tumor cells and of the number of lung metastases. These adverse effects of radiation were prevented by **downregulating** the MT1-MMP.

Conclusions: This preclinical model of TNBC suggests that a minimum level of MT1-MMP was necessary for the stimulation of lung metastases caused by radiation, and that its expression level and/or localisation could be evaluated as a biomarker for predicting the early recurrence observed in some TNBC patients.

INTRODUCTION

Triple negative breast cancer (TNBC– receptors estrogen, progesterone and Her2 negative) represents 10-20% of all breast carcinomas and is characterised by a lack of estrogen, progesterone and human epidermal growth factor receptors (Podo *et al*, 2010). Compared to non-TNBC, TNBC is associated with a higher risk of locoregional recurrence, metastasis development and greater mortality (Mahamodhossen *et al*, 2013). It is one of the poorest prognosis breast cancer subtype, and to date, there is no clinically available targeted therapy (Zhou *et al*, 2013).

Radiotherapy is considered for all patients who have had breast conservative surgery. After excision of the primary tumor with negative margins, microfoci of cancer cells scattered throughout the breast are frequently left behind, as revealed by detailed serial sectioning of mastectomy specimens (Roland *et al*, 1985). These microfoci will be targeted using chemotherapy and radiotherapy where the whole breast is irradiated. There is no doubt concerning the net benefit of radiotherapy since it reduces the rate of locoregional and distant recurrence (Tamaki *et al*, 2013). However, radiotherapy does not always eradicate all cancer cells scattered throughout the breast and increasing radiation doses is not a viable option as it may cause undesired long term complications to normal tissues (Yi *et al*, 2009).

Some TNBC patients benefit from radiotherapy and are cured. Conversely, for other TNBC patients, metastases appear during the first 3 years after treatment and cure is unlikely (Podo *et al*, 2010). Since there is no biomarker or other relevant clinicopathological factors to identify TNBC at high risk of early recurrence, these patients are treated with standard treatments and results are mostly disappointing (Pogoda *et al*, 2013). Identification of new biomarkers predicting the risk of early recurrence would significantly improve the clinical management of TNBC.

The ability of radiation to stimulate cancer cell invasion and metastasis development was observed: 1) after irradiation of tumors implanted in animal models (Sofia Vala *et al*, 2010; Jia *et al*, 2011; Chou *et al*, 2012), 2) by irradiating healthy tissue before implantation

of nonirradiated cancer cells (Lemay *et al*, 2011; Bouchard *et al*, 2013; Desmarais *et al*, 2015) and 3) when cancer cells were irradiated before their implantation in animals (Wild-bode *et al*, 2001). Interestingly, this adverse effect of radiation is not observed in all cancer cells, but only in some subtypes (Wang *et al*, 2000; Wild-bode *et al*, 2001; Qian *et al*, 2002; Rofstad *et al*, 2004; Speake *et al*, 2005; Paquette *et al*, 2011; Jiang *et al*, 2014). In a mouse model, irradiation of the mammary gland before the implantation of murine TNBC cells resulted in an increase of cancer cell invasion and lung metastasis development (Bouchard *et al*, 2013). The general belief regarding radiation-induced genetic mutations leading to malignancy and increased invasive phenotype was excluded in this study since cancer cells did not receive any radiation (Rodemann & Blaese, 2007; Barcellos-Hoff, 2013; Bouchard *et al*, 2013). Considering that a subgroup of TNBC patients could potentially be resistant to radiotherapy and will rapidly develop recurrence (Podo *et al*, 2010; Langlands *et al*, 2013), it is relevant to determine whether early recurrence in these TNBC patients is related to the ability of radiation to stimulate metastasis development.

Stimulation of cancer progression induced by radiation has been suggested to be associated with inflammation that is triggered in all patients (Rodemann & Blaese, 2007; Bouchard *et al*, 2013). Radiation-induced inflammation in normal tissue is characterised by an increase of cytokines and matrix metalloproteinases (MMPs) (EBCTCG *et al*, 2005; Rodemann & Blaese, 2007). MMPs is a family of more than 20 endopeptidases that cleave various substrates of the extracellular matrix (ECM) and have been associated with breast cancer invasion (Egeblad & Werb, 2002). Among them, MMP-2 was previously suggested to be involved in radiation-enhancement of cancer progression (Lemay *et al*, 2011; Desmarais *et al*, 2012). However, proMMP-2 and -9 need to be activated by the protease MT1-MMP expressed on the surface of cancer cells. Consequently, invasion of cancer cells is dependent on the expression of MT1-MMP (Hotary *et al*, 2002).

The main activator of MMP-2 is the membrane-type matrix metalloproteinase-1 (MT1-MMP) that can also degrade ECM macromolecules including collagen, fibronectin, vitronectin, fibrin and aggrecan by localising at the leading edge of the cell (Itoh, 2006). High levels of MT1-MMP were correlated with blood vessel invasion of cancer cells in

TNBC patients (Perentes *et al*, 2011) and were also associated with shorter overall survival (Li *et al*, 2015). MT1-MMP blockade with a specific antibody in a preclinical mouse model decreased the growth of 4T1 TNBC tumors and synergistically enhanced tumor response to radiation therapy (Ager *et al*, 2015).

Although residual tumor cells potentially left in the breast after partial mastectomy are targeted by radiation, we decided to assess the role of the MT1-MMP in TNBC cells by irradiating the mammary gland of BALB/c mice before the implantation of cancer cells. As tumor biopsies used to establish the diagnosis and prognosis are analysed before radiotherapy, this experimental procedure excludes the possibility that new mutations induced by radiation would have modified the invasiveness of TNBC cells. This model also allows to better understand the association between radiation-induced inflammation in the mammary gland, the level of MT1-MMP in TNBC cells and the development of lung metastases.

MATERIALS AND METHODS

Cell culture

The mouse breast carcinoma D2A1 cells, kindly provided by Dr. Ann F. Chambers (University of Western Ontario), were derived from a spontaneous mammary tumor in a BALB/c mouse and the mouse 3T3 fibroblasts were obtained from the American Type Culture Collection (ATCC). TNBC status of the D2A1 cells was confirmed by a certified pathologist, Dr. Sameh Geha from the Centre Hospitalier Universitaire de Sherbrooke, using standard immunohistochemistry protocols suitable for the detection of mouse estrogen receptor (ER), progesterone receptor (PR) and HER2. Cells were maintained in a 5% CO_2 humidified incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Wisent), 2 mM glutamine, 1 mM sodium pyruvate, 100 units per mL penicillin and 100 mM streptomycin.

Downregulation of MT1-MMP expression by short hairpin RNA (shRNA)

For the production of lentiviral particles, the human cell line 293T were co-transfected using lipofectamine with 6 μg of the plp1, plp2, and plp/VSV-G plasmids (Invitrogen) and 6 μg of pLKO.1-puro vector containing a shRNA sequence targeting murine MT1-MMP transcript (Sigma-Aldrich: TRCN0000031264, TRCN0000031265, TRCN0000031266, TRCN0000031267). The pLKO empty vector was used as a negative control (mock cells). After 48 h, the lentivirus-containing supernatant (cell media) was collected and filtered with a 0.45 μm membrane and then kept at −80°C for further use. D2A1 cells expressing the fluorescent proteins FUCCI were prepared as previously described (Bouchard *et al*, 2013). The D2A1 FUCCI cells were infected by with 700 μl of the virus-enriched supernatant containing 4 μg/ml polybrene (Sigma-Aldrich). After an incubation of 48 h, DMEM containing virus was replaced with fresh DMEM containing 1.5 μg/ml of puromycin as selection agent and incubated for 10 days.

Validation scheme for down-regulation of MT1-MMP is described in Supplementary Methods. Briefly, quantitative Polymerase Chain Reaction (qPCR) was used to confirm MT1-MMP mRNA (*Mmp14*) knockdown (Fig. S1A), and the decrease in MT1-MMP protein level was confirmed by Western blot (Fig. S1B and C). Reduction of MMP-2 activation, the main extracellular substrate of MT1-MMP, was shown by zymogram analysis (Fig. S1D and E). Nomenclature of the derived D2A1 cell lines is as following: D2A1-wt (wild-type), D2A1 shMT1-mock (pLKO empty vector), D2A1 shMT1-40 (40% downregulation of the Mmp14 transcript level) and D2A1 shMT1-70 (70% downregulation of the Mmp14 transcript level).

Invasion capacity of D2A1 cells assessed in invasion chambers

Invasion capacity of D2A1 cells expressing different levels of MT1-MMP was assessed in invasion chambers as previously described (Bouchard *et al*, 2013). Briefly, BALB/c 3T3 fibroblasts ($2.5x10^4$) were seeded with MEM supplemented with 10% FBS in 24-well plates. After 20 h, the cell culture medium was replaced with MEM supplemented with 0.1% bovine serum albumin (BSA) following two rinses in PBS. Cells were then irradiated using a ^{60}Co source (Gammacell 220, Nordion) at a dose of 5 Gy. Sham irradiated cells

were used as a control. The fibroblast conditioned media were used as a chemoattractant in the lower compartment of the invasion chambers (Becton Dickinson Biosciences). Invasion chambers coated with Matrigel (artificial basement membrane) were rehydrated with 1 ml MEM 0.1% BSA for 2 h at 37°C. Nonirradiated D2A1 cells harvested with Cell Dissociation Solution (Sigma-Aldrich) were added ($4x10^4$) to the upper compartment of the invasion chambers 24 h after irradiation of the BALB/c 3T3 cells. D2A1 cells that had passed across the Matrigel and the porous membrane 24 h later were fixed, stained with crystal violet and counted under the microscope. The invasion capacity of the MT1-MMP wild-type and downregulated cells were compared. Results were reported as radiation-enhancement ratio. Each experiment was performed in triplicate and repeated 2 times.

Mammary gland irradiation

The experimental protocols were approved by the Université de Sherbrooke Ethics Committee for Animal Care and Use in accordance with guidelines established by the Canadian Council on Animal Care (Protocol ID number 013-14). Female retired breeder BALB/c mice (18–24 weeks old) were obtained from Charles River. Animals were anaesthetised with 3% isoflurane and then immobilised with a stereotactic mouse frame adapted to dock on the Leskell Gamma Knife Perfexion (Elekta). The third right mammary gland was irradiated by an energy deposition of elliptical shape (Bouchard *et al*, 2013). Anaesthetised mice were irradiated daily with 6 Gy for a total of 4 fractions. Based on dosimetry performed by our institutional medical physicist team, this protocol provided a biological effective dose (BED) comparable to the standard clinical regimen of 20 x 2.25 Gy, without having to perform daily anesthesia over 20 days that can be lethal for mice. Regarding the third left mammary gland (nonirradiated control) in the same animal, a residual dose of < 1% was received.

Implantation of D2A1 FUCCI expressing cells in mammary glands

D2A1 FUCCI-expressing cells (10^6 per 100 µl PBS) expressing different levels of MT1-MMP were implanted in preirradiated (right side) and in the opposite nonirradiated (control; left side) mammary glands 3 h after the last irradiation. These D2A1 cells were also implanted into the mammary glands of sham-irradiated mice, and the number of their

circulating tumor cells and lung metastases were compared to those of irradiated animals. Overall, 8 groups of mice were assessed; SHAM + D2A1-wt, IRR + D2A1-wt, SHAM + D2A1 shMT1-mock, IRR + D2A1 shMT1-mock, SHAM + D2A1 shMT1-40, IRR + D2A1 shMT1-40, SHAM + D2A1 shMT1-70 and IRR + D2A1 shMT1-70. To exclude possible systemic effects of radiation on tumor growth, tumor volumes of sham-irradiated animals (sham tumors) were compared to control tumors (left side) of irradiated animals as a validation of the mice as its own control in following experiments (Fig. S2). Tumor volumes were measured every 3 days using a caliper and calculated with the formula: V (mm^3) = $\pi/6$ x a (mm) x b^2 (mm^2), where 'a' and 'b' are the largest and smallest perpendicular tumor diameters.

Quantification of pulmonary metastases by optical imaging

Fluorescence emitted by D2A1 FUCCI-expressing cells was used to quantify the number of lung metastases with an animal optical imager (QOS Imager; Quidd S.A.S.). On day 21, mice were euthanised, and lungs were removed. A bright field image of the lungs was taken and then the appropriate filters were selected for red and green fluorescent image acquisition (mKO2, λex = 472/30, λem = 536/40; mAG, λex = 531/40, λem = 593/40). The three images acquired were merged for quantification of lung metastases.

Histology

Mammary tumors and lung specimens containing D2A1 FUCCI-expressing cells were dissected and frozen in optimal cutting temperature compound (OCT; Electron Microscopy Sciences) for cryosections or fixed with 4% paraformaldehyde (PFA) before paraffin embedding. After H&E staining, primary tumor and infiltrative areas in fixed specimens sections were delimited microscopically with a Nanozoomer (Hamamatsu) using Nanozoomer Digital Pathology software. Migration ratio were quantified as Infiltrative area/Primary tumor area.

Cryosections of 3 µm were cut using a Leica CM3050 Microsystems cryostat (Wetzlar). Slides were dried for 30 min at 37°C and then stored at -80°C until further use. The fluorescence emitted by the D2A1 cells was recorded using the FSX100® Bio Imaging

Navigator microscope (Olympus) equipped with band pass filters (Chroma Technology Corp) for fluorescein isothiocyanate (FITC (green); λex = 480/30, λem = 535/40) or tetramethylrhodamine isothiocyanate (TRITC (red); λex = 560/40, λem = 630/60). The entire tumor slide was scanned (magnification X 42) and the ratio of red and green fluorescence intensity emitted by tumor cells were calculated. Immunohistochemistry (IHC) was performed as described in Supplementary Methods.

Circulating tumor cells (CTC)

Blood samples were collected from the lateral saphenous vein of the sham and irradiated mice at days 4 and 7 after the implantation of D2A1 FUCCI-labeled cells expressing different levels of MT1-MMP in the mammary glands. Samples diluted 1:10 in PBS were spread in a Petri dish and covered with a glass cover slip for quantification. The presence of CTC in each blood sample was quantified by fluorescence microscopy from 5 images of representative areas (magnification X 100) that were acquired as described above with an Olympus fluorescence microscope. Fluorescence microscopy method was chosen over FACS analysis, allowing repeated quantifications with small blood volumes.

Statistical analysis.

Experimental data are shown as mean ± standard error mean (SEM). Statistical analyses were performed using one or two-way analysis of variance (ANOVA) with multiple comparisons Fisher's least significant difference (LSD) test where each comparison stands alone. A P value of 0.05 was considered to be statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

RESULTS

MT1-MMP promotes radiation-stimulated invasion *in vitro*

To determine whether MT1-MMP is involved in radiation-stimulated invasion of D2A1 breast cancer cells, two stable cell lines expressing lower levels of MT1-MMP were prepared using shRNA. Downregulation of 40% and 70% were achieved as assessed by quantifying mRNA by qPCR (Fig. S1A). Protein expression was also quantified by

Western blot analyses (Fig. S1B and C) and reduction of the enzymatic activity of MT1-MMP was confirmed by measuring the conversion of proMMP-2 to MMP-2 by zymography (Fig. S1D and E). A third cell line was generated with the empty plasmid as a negative control (mock). Cell lines used in this study were identified as D2A1-wt (wild-type), D2A1 shMT1-mock (pLKO empty vector), D2A1 shMT1-40 (40% downregulation of MT1-MMP) and D2A1 shMT1-70 (70% downregulation of MT1-MMP).

The invasiveness of these cell lines was assessed with invasion chambers coated with Matrigel™, and using the BALB/c 3T3 fibroblasts irradiated with 5 Gy as chemoattractant in the lower compartment of the chamber. The invasiveness of D2A1-wt was increased by 1.6-fold compared to assays done with nonirradiated 3T3 cells ($P < 0.0001$) (Fig. 1A). Reduction of MT1-MMP expression decreased the basal invasion capacity of the D2A1 cells. Invasiveness of the D2A1 shMT1-40 was reduced by 32% ($P = 0.0108$) and by 63% for the D2A1 shMT1-70 cells ($P < 0.0001$). More significant, the ability of radiation to promote the invasion of D2A1 cells was completely inhibited for both MT1-MMP downregulated cell lines. These results support the important role of MT1-MMP in invasion as well as radiation-stimulated invasion of D2A1 breast cancer cells *in vitro*.

MT1-MMP expression affects growth of D2A1 tumor implanted in preirradiated mammary gland only

The D2A1 cell lines were implanted in mammary glands of BALB/c mice that were preirradiated or not (controls). No difference in tumor volumes was measured with the four cell lines (D2A1-wt, shMT1-mock, shMT1-40 and shMT1-70) when implanted in nonirradiated mammary gland (solid lines) (Fig. 1B). On the other hand, 3 weeks after implantation, tumor volumes were significantly decreased for these four cells lines when implanted in preirradiated mammary gland (dotted lines) ($P < 0.0001$), a reduction that was more important for the MT1-MMP downregulated tumors (Fig. 1B). Although the shMT1-40 tumors seemed to be smaller than the shMT1-70 tumors, their respective volumes were not statistically different ($P = 0.1819$). Reduction of tumor growth in preirradiated mammary glands was not associated with a decrease of mitotic activity since no significant

difference in Ki67 positive cells (proliferation marker) was measured between all D2A1 tumors (Fig. 1C and D).

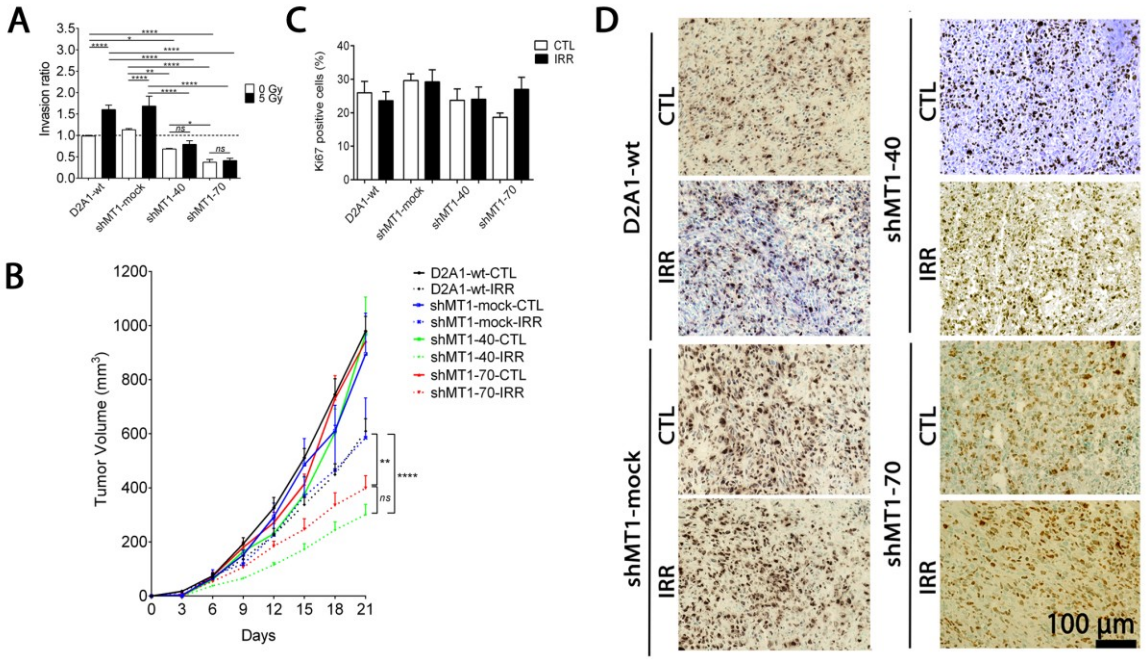

Figure 1. Effect of the preirradiated microenvironment on D2A1 breast cancer cell invasion and tumor growth assessed with cell lines downregulated for MT1-MMP. **(A)** Conditioned media from irradiated (5 Gy) 3T3 fibroblasts was added in the lower compartment of the invasion chambers and used as chemoattractant for breast cancer cells added in the upper compartment. MT1-MMP downregulation completely blocked radiation-enhancement of invasion in TNBC cell lines. The downregulation of the MT1-MMP also reduced the basal invasiveness of D2A1 cells. Decreases of 32% and 63% were observed for D2A1 shMT1-40 and D2A1 shMT1-70 cells ($P < 0.0001$). The invasion assays were done in triplicate and repeated 2 times. **(B)** D2A1 tumor volumes measured after implantation in preirradiated (dotted lines) or nonirradiated (solid lines) mammary glands of BALB/c mice. At day 21, tumor volumes for all cell lines were significantly reduced when implanted in preirradiated mammary glands, compared to nonirradiated mammary glands. Downregulation of MT1-MMP expression further reduced the tumor volume, but only in the preirradiated tissue. Volumes of shMT1-40 and shMT1-70 tumors were 2-fold and 1.5-fold decreased compared to wild-type tumors. n = 9 to 21 tumors for each group. **(C)** Quantification of Ki67 positive cells by IHC on tumor frozen sections. No difference was observed. $n = 4$ to 6 tumors for each group. **(D)** Representative IHC images of Ki67 staining. CTL = control, IRR = irradiated. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$.

MT1-MMP downregulation decreases tumor vascularisation

Modifications of vascularisation can affect tumor growth and invasion of cancer cells. The impact of MT1-MMP downregulation on tumor vascularisation was then assessed by quantifying the endothelial cell marker CD31 by IHC on tumor frozen sections. A significant decrease by 36 % ($P < 0.0001$) of CD31 staining was measured only in the D2A1 shMT1-70 tumors (Fig. 2). A reduction of CD31 staining was also observed in the D2A1 shMT1-40 tumors, but the decrease was not statistically significant. The diminution of blood vessel area was independent of the preirradiated microenvironment since no difference was observed between control tumors (left side) and those implanted in preirradiated mammary glands (right side).

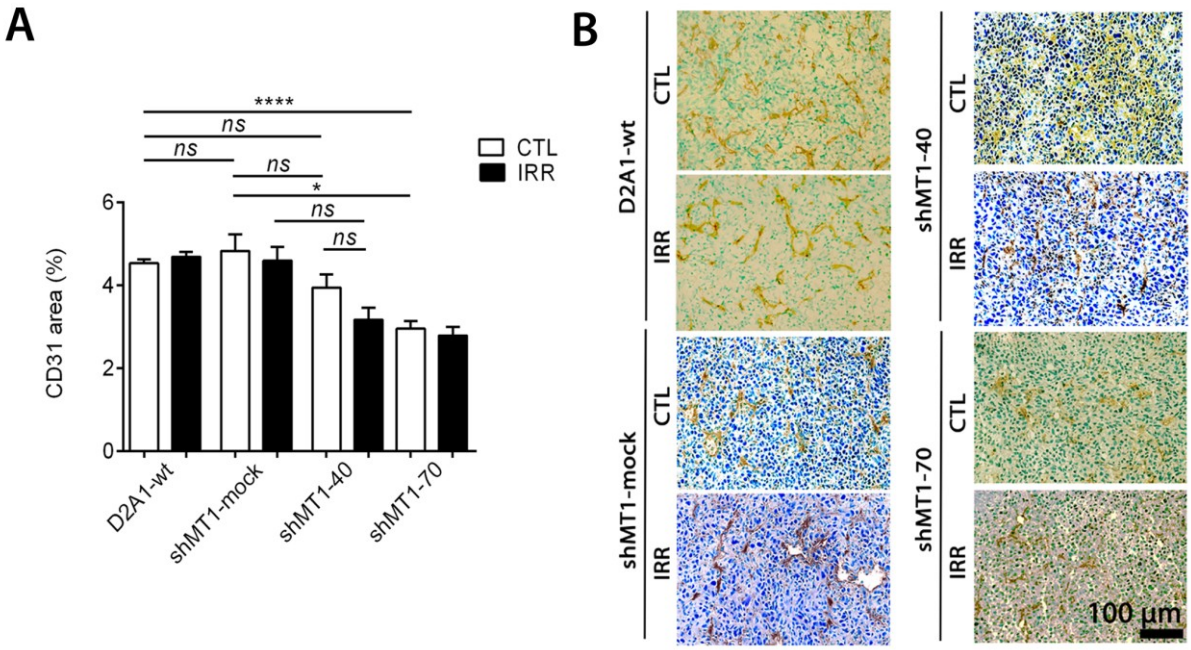

Figure 2. Vascularisation assessed in D2A1 tumor frozen sections. **(A)** Quantification of the blood vessel marker CD31 plotted as percentage of stained area. Mammary gland irradiation did not significantly modify the CD31 staining in tumors, except for the shMT1-70 tumors in which a significant reduction was measured (n = 4 to 6 for each). **(B)** Representative IHC images of CD31 staining. CTL = control, IRR = irradiated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Hypoxia inducible factor 1 alpha (HIF-1α) is a direct effector of hypoxia that induces the recruitment of endothelial progenitor cells to promote neovascularisation (Du *et al*, 2008). Stabilisation of HIF-1α in the nucleus indicates ongoing hypoxic activity as well as its presence in the cytoplasm since this protein is synthesised and degraded in this compartment (Giatromanolaki *et al*, 2006). The effect of MT1-MMP downregulation on HIF-1α activity was then assessed by IHC on D2A1 tumors sections. HIF-1α was found in the nucleus as well as in the cytoplasm of cancer cells and significant modifications in the protein expression were measured for MT1-MMP knockdown tumors implanted in preirradiated mammary glands compared to nonirradiated mammary glands. The mean HIF-1α signal for shMT1-40 tumors was increased by 1.6-fold ($P = 0.0016$) and by 1.7-fold ($P = 0.0004$) for shMT1-70 tumors implanted in irradiated mammary glands suggesting that the preirradiated microenvironment promoted hypoxia in knockdowns tumors (Fig. 3A, B).

Preirradiation of mammary gland modifies the MT1-MMP localisation in tumor cells

The diminution of MT1-MMP did not affect the morphology of the D2A1 cells, neither implanted in control nor preirradiated mammary glands (Fig. 3C, D). MT1-MMP was mainly localised in nuclei (black arrowheads) and cytoplasm but perinuclear staining was also observed in some cells (arrows). Interestingly, preirradiation of the mammary glands has modified MT1-MMP localisation in downregulated tumors, as shown by a lower accumulation in nucleus (Fig. 3D). The number of negative nucleus was increased by 2.9 times ($P < 0.0001$) for both shMT1-40 and shMT1-70 tumors implanted in preirradiated mammary gland compared to nonirradiated controls. This reduction of MT1-MMP accumulation in cancer cell nucleus was not observed with the D2A1-wt and mock tumors implanted in the preirradiated mammary glands. It should be noted that MT1-MMP is also expressed in the normal mammary epithelium but its endogenous expression was not affected by radiation (Fig. S3). Normal mouse mammary tissue was also used as a positive control for the MT1-MMP antibody used in this study (Fig. S4).

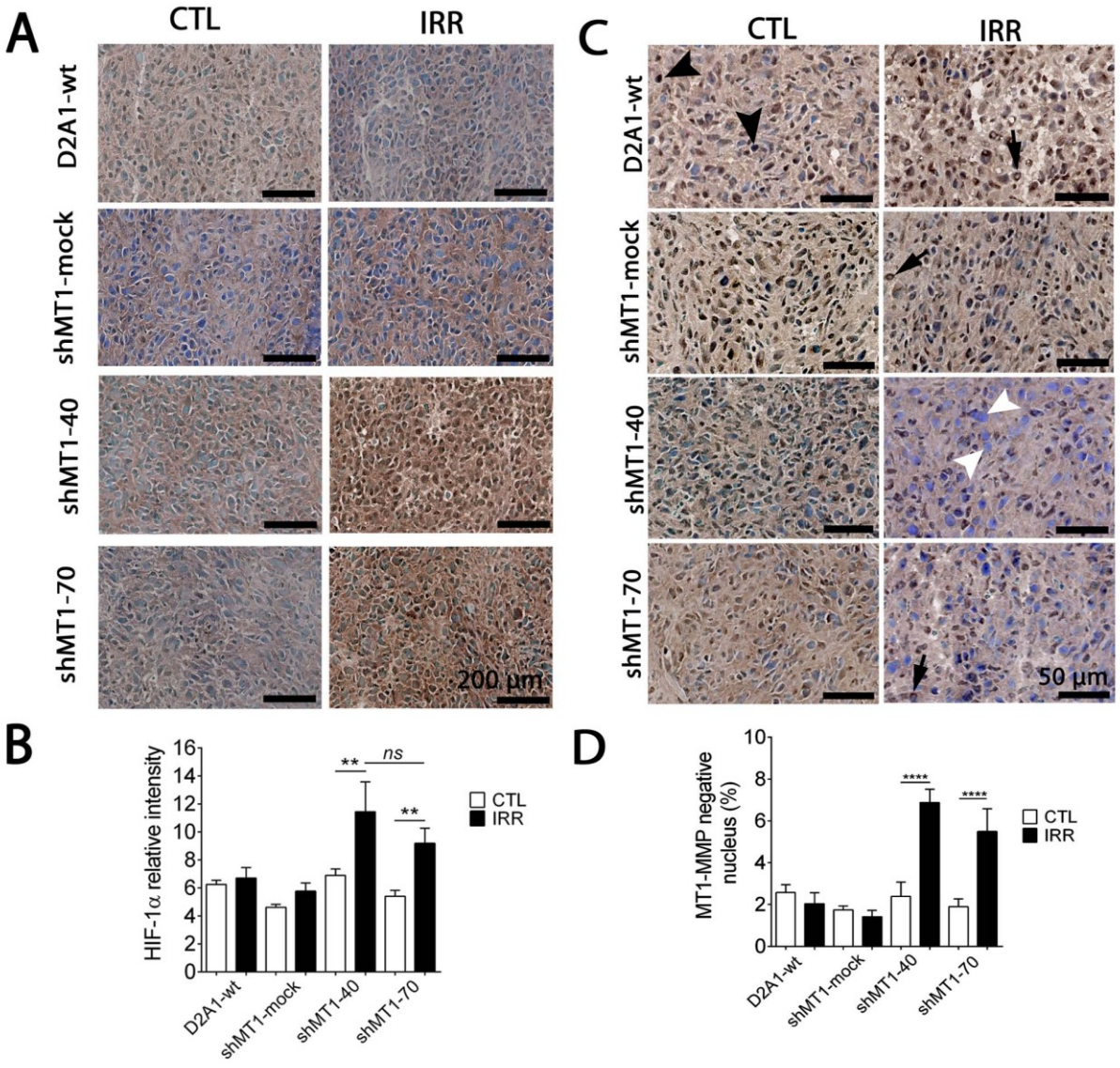

Figure 3. Hypoxia quantification assessed by HIF-1α and MT1-MMP localisation on tumor sections. **(A)** Representative IHC images of HIF-1α staining in frozen tumor sections. **(B)** Mean intensity of HIF-1α staining from 5 representative areas for each conditions. Hypoxia was significantly increased only for shMT1-40 (1.6-fold) and shMT1-70 (1.7-fold) tumors implanted in preirradiated mammary glands (n = 2 to 5 tumors for each condition). **(C)** Representative IHC images of MT1-MMP staining in frozen tumor sections. (n = 3 tumors for each group). **(D)** Quantification of the MT1-MMP negative nucleus. Black arrowheads = MT1-MMP positive nuclei, white arrowheads = MT1-MMP negative nuclei, arrows = perinuclear staining. CTL = control, IRR = irradiated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Reduction of MT1-MMP expression prevents radiation-induced of CTC and lung metastases

Our previous study showed that the number of CTC and lung metastases were significantly increased when the D2A1-wt cells were implanted in a preirradiated compared to a nonirradiated microenvironment (Bouchard *et al*, 2013). To determine the role of MT1-MMP in cell dissemination, CTC were quantified on day 4 and 7 after the implantation of D2A1 shMT1-40 and shMT1-70 cells in the mammary fat pads (Fig. 4A). Furthermore, lung metastases were quantified by optical imaging on day 21 post-implantation of the tumors (Fig. 4B and C). The irradiation promoted the migration of D2A1 cells from the mammary gland to the blood vessels as showed by higher number of CTC when the D2A1-wt and shMT1-mock cells were implanted. This increase was statistically significant at day 7 only (2.5 times; $P < 0.0001$, 2.4 times; $P = 0.009$). Conversely, the number of CTC was not increased when the MT1-MMP-deficient cells were implanted in irradiated mice compared to sham irradiated mice.

The increase of CTC induced by radiation was correlated with a higher number of lung metastases when mice were implanted with D2A1-wt or shMT1-mock cells ($P = 0.0002$; $P = 0.0048$) (Fig. 4B and C). As expected, this stimulation by irradiating the mammary gland was not observed when the MT1-MMP-deficient cells were implanted. It is noteworthy that reduction of MT1-MMP expression did not significantly decreased basal development of lung metastases since the number of metastases measured in the sham irradiated mice implanted with the D2A1 shMT1-40 or shMT1-70 cells was similar to those measured after implanting the D2A1-wt or mock cells. Moreover, there was no significant difference in the number of lung metastases between the cell lines displaying either 40% or 70% reduced MT1-MMP expression.

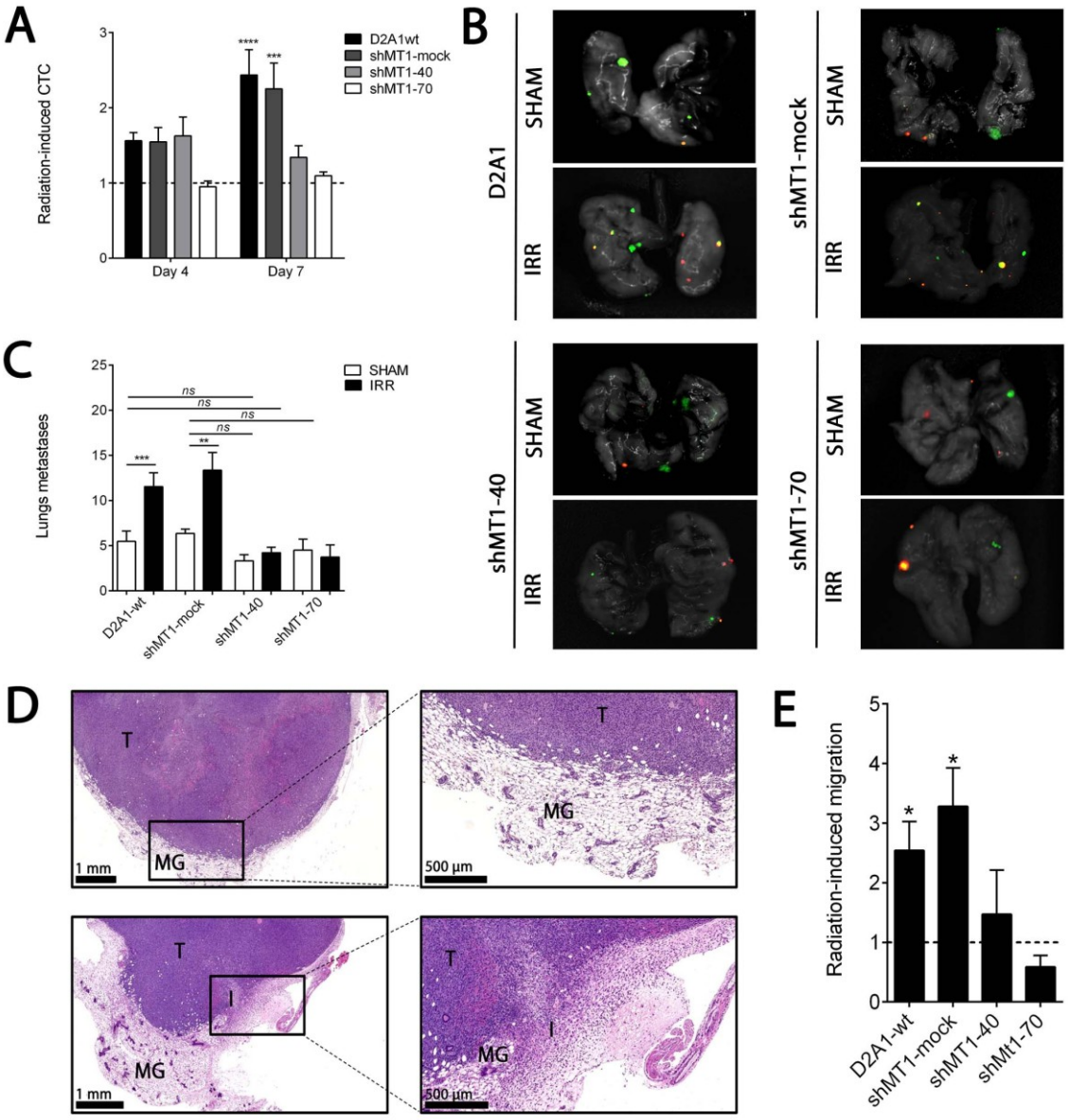

Figure 4. Downregulation of MT1-MMP expression has inhibited radiation-enhancement of cancer cell invasion, CTC and lung metastases. **(A)** Quantification of CTC in blood samples of sham and irradiated mice. Results were plotted as radiation-enhancement of CTC number. The increase of CTC number was statistically significant only on day 7 for D2A1-wt and shMT1-mock cells. This radiation-enhancement of CTC number was not observed in the shMT1-40 and shMT1-70 cells implanted in mice. $n = 3$ to 11 for each condition. **(B)** Optical imaging of lung metastases on sacrifice day, 21 days after tumor implantation. **(C)** Quantification of the number of lung metastases. The number of lungs metastases was increased in irradiated mice compared to sham groups for D2A1-wt and shMT1-mock cells. This radiation-enhancement of lungs metastases was not observed with the shMT1-40 and shMT1-70 cells ($n = 8$ to 23 for each condition). Sham mice: nonirradiated animals with tumor implantation in the right and left third mammary glands. Irradiated animals: preirradiation of the third right mammary gland followed by

tumor implantation in the right and left third mammary glands. **(D)** H&E staining of D2A1 tumor sections showing that radiation-induced migration in mammary glands was MT1-MMP-dependent. **(E)** Quantification of D2A1 tumor migration. Migration ratios were calculated as infiltration area divided by primary tumor area. Migration ratios were higher in tumors implanted in preirradiated mammary glands for the D2A1-wt and shMT1-mock tumors. This radiation-enhancement of migration was not observed in the shMT1-40 and shMT1-70 tumors (n = 2 to 6 for each condition). T = D2A1 tumo, MG = mammary gland, I: Infiltration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Effect of MT1-MMP on cell cycle distribution

Cancer cells can either migrate or proliferate, a phenomenon known as the migration/proliferation dichotomy (Giese *et al*, 1996) . In our model, the FUCCI colorimetric vectors (Sakaue-Sawano *et al*, 2008) expressed by the D2A1 cells generate a green fluorescence when cells are in the $S/G_2/M$ phases (dividing cells) and red fluorescence for the G_1/G_O phases (non-dividing enriched cells). Since MT1-MMP downregulation prevented radiation-enhancement of CTC and lung metastases, we determined whether this stimulation was associated with enrichment to the G_1/G_O phases, which would favour cancer cell migration. Preirradiation of mammary glands increased D2A1 cells in the G_1/G_O phases by 20% ($P < 0.0001$) for D2A1-wt and shMT1-mock tumor cells (Fig. 5). This increase of cells in G_1/G_O phases (red cells) was associated with a decrease of dividing cells (green cells). Promotion in the G_1/G_O phases induced by irradiated mammary gland was not observed in D2A1 cells downregulated for MT1-MMP.

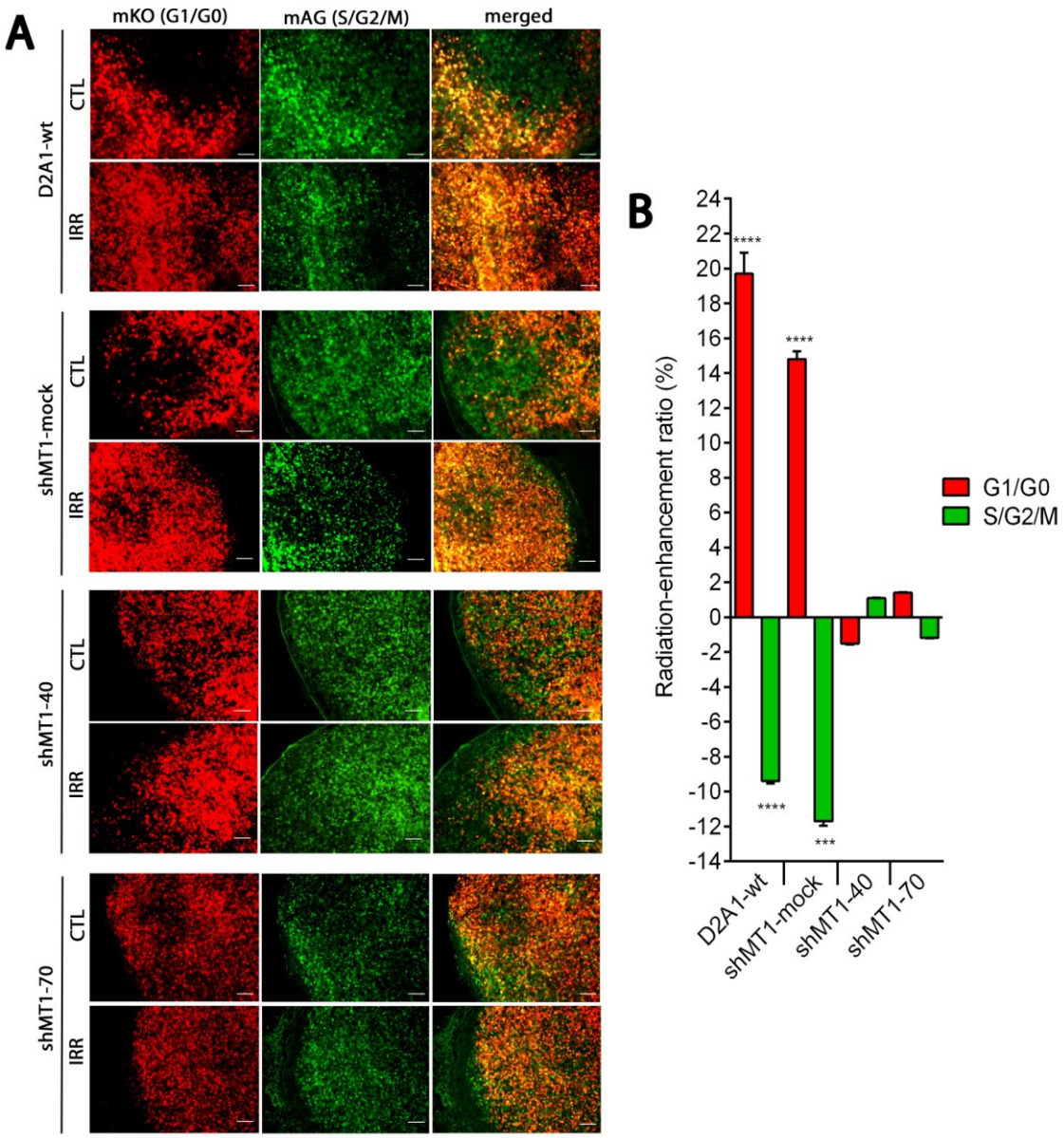

Figure 5. Effect of MT1-MMP downregulation on cell cycle distribution in D2A1 FUCCI tumors. **(A)** Representative fluorescence images of frozen mammary tumor sections that were used to quantify cancer cells in S/G2/M (green) or G1/G0 (red) phases (magnification X 42, scale bar = 200 μm). **(B)** Effect of radiation on cell cycle distribution plotted as radiation-enhancement ratio of red and green cells in percentage. A significant radiation-enhancement of G1/G0 (red) cells was observed for D2A1-wt and shMT1-mock tumors. MT1-MMP downregulation completely prevented this augmentation (n = 6 to 13 for each condition). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Association between an enrichment in the phases G_1/G_O and promotion of the invasion phenotype was then assessed in tumor paraffin-embedded sections after H&E staining. Primary tumors and infiltration areas were measured for each condition. In D2A1-wt and mock tumors implanted in nonirradiated mammary glands, no significant migration to the healthy tissue was observed (Fig. 4D, upper panel). As expected, migration ratios were increased in D2A1-wt (P = 0.0220) and shMT1-mock tumors (P = 0.0433) implanted in preirradiated mammary glands compared to nonirradiated mammary glands (Fig. 4D, bottom panel). On the other hand, migration of D2A1 cells in the irradiated mammary glands was blocked when D2A1 cells expressing reduced levels of MT1-MMP were implanted (Fig. 4E).

Overall, these results support the important role of MT1-MMP in radiation-stimulated invasion in a mouse model of D2A1 triple negative breast cancer cells, as summarised in Fig. 6.

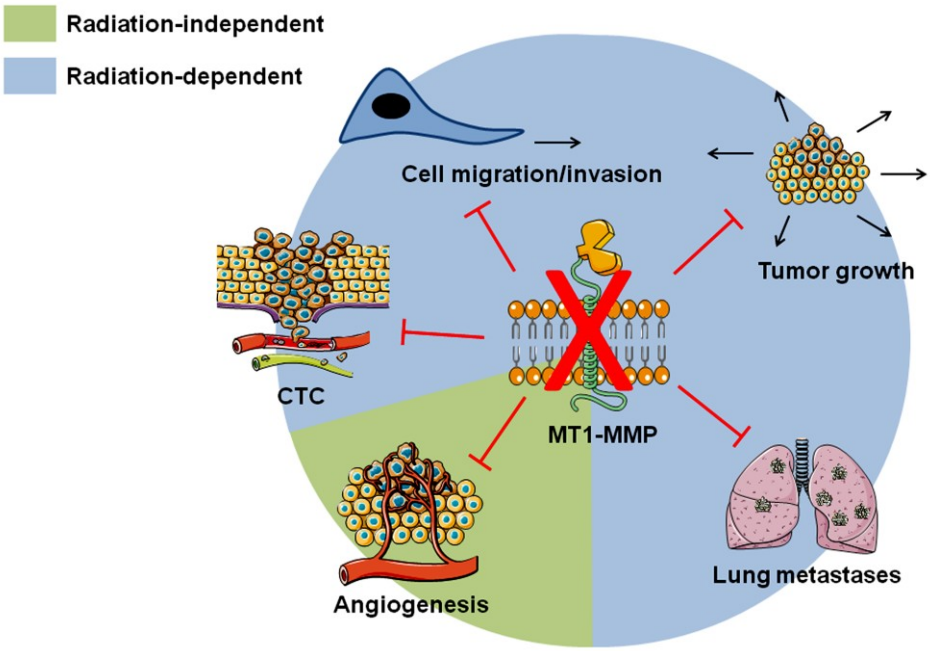

Figure 6. Summary of the proposed involvement of MT1-MMP in radiation-stimulated development of lung metastases.

DISCUSSION

The concept of radiation-stimulated cancer cell migration and metastasis development has recently emerged (Desmarais *et al*, 2012; Bouchard *et al*, 2013; Moncharmont *et al*, 2014). The present study highlights the contribution of the MT1-MMP in the promotion by radiation of cancer cell invasion and formation of lung metastases in an animal model of TNBC. This undesired effect of radiation was not caused by an induction of new mutations in D2A1 cells since they were not irradiated. Our results support that this MT1-MMP-related effect was rather associated with the irradiation of the mouse mammary gland. In addition, these results suggest that analysis of MT1-MMP status in tumor biopsies prior to radiotherapy would be appropriate to assess the diagnosis and prognosis.

Since only the D2A1 cells were assessed, this role of MT1-MMP in radiation-stimulated metastasis development should be validated with other TNBC cell lines. Using *in vitro* models, the ability of radiation to increase the invasiveness of human breast cancer cell lines was previously demonstrated (Paquette *et al*, 2011, 2013; Bouchard *et al*, 2016). For animal models, syngenic mouse, as used in our study, are more appropriate because radiation-induced lung metastasis development is associated with an inflammatory response. Therefore, since human xenografts need to be implanted in immunodeficient mice, the promotion of cancer cell invasion and metastasis development by radiation would not be properly assessed using these animal models.

Until now, the role of MT1-MMP in human TNBC cell lines was assessed *in vitro* and in animal models only in nonirradiated conditions. A downregulation of the MT1-MMP reduced the invasion of the human TNBC cell line MDA-MB-231 *in vitro* and decreased the number of lung metastases (Jiang *et al*, 2006; Perentes *et al*, 2011). Using human biopsies from TNBC, elevated MT1-MMP expression was correlated with blood vessel invasion of cancer cells, a poor prognosis and high incidence of distant metastasis, relative to other breast cancer subtypes (Perentes *et al*, 2011). It remains to be determined whether radiotherapy can promote cancer cell invasion and metastasis development in TNBC patients at high risk of early recurrence.

Using a D2A1 TNBC model, irradiation of the mouse mammary gland has increased the number of migrating cells. This migration/proliferation dichotomy was described as mutually exclusive or as a «Go or Grow» phenomenon, and it can be induced by radiation (Giese *et al*, 1996; Desmarais *et al*, 2012; Bouchard *et al*, 2013). Consequently, an enrichment of migrating cells induced by radiation could explain the reduction of tumor growth when the D2A1-wt and mock cells were implanted in the preirrradiated mammary glands. However, reduction of tumor growth was more important in the MT1-MMP downregulated tumors, although radiation-enrichment of migrating D2A1 cells was inhibited in these tumors. On the other hand, the downregulation of MT1-MMP did not affect tumor volume when the D2A1 shMT1-40 and shMT1-70 cells were implanted in nonirradiated mammary glands. Similar results were reported by other groups when comparing wild-type with MT1-MMP deficient MDA-MB-231 cells in nonirradiated conditions (Jiang *et al*, 2006; Perentes *et al*, 2011). Reduction of tumor growth in the preirrradiated mammary glands can thus not be solely associated with a downregulation of the MT1-MMP.

Since MT1-MMP expression can promote angiogenesis (Sounni *et al*, 2002), we have determined whether a decrease of angiogenesis could explain the reduction of tumor growth in irradiated mammary glands. For the D2A1-wt and mock tumors, no reduction of the CD31 area was measured although their growth was reduced in irradiated mammary glands. Since a tumor growth reduction was measured only in irradiated mammary glands, angiogenesis does not seem to play a significant role in our model as the CD31 area in MT1-MMP downregulated tumors was reduced to similar levels for both tumor groups, either implanted in irradiated or nonirradiated mammary glands.

Our study has also contributed to further understand the effect of radiation on blood vessels and its impact on the increase of CTC. Preirradiation of the mammary gland increased the number of CTC after D2A1-wt and mock cell implantation. This stimulation cannot be associated with significant radiation-induced blood vessel damages since downregulation of MT1-MMP completely inhibited the radiation-increased number of CTC. These results suggest that the radiation dose used in our study did not induce

damages to blood vessels that would significantly facilitate the entry of cancer cells into the bloodstream.

The predictive value of MT1-MMP to identify TNBC patients at high risk of early recurrence could depend on its level of expression and its localisation in tumor cells. Elevated expression of MT1-MMP in breast tumor biopsies was previously associated with distant metastasis and poor prognosis (Perentes *et al*, 2011; Li *et al*, 2015). In our study, MT1-MMP mRNA, protein expression and enzymatic activity by measuring the activation of proMMP-2 to MMP-2 were quantified. MT1-MMP mRNA was downregulated by 40% and 70% respectively in the D2A1 shMT1-40 and shMT1-70 cells homogenates but the protein expression reached 18% and 40% reduction, as quantified in tumor homogenates. On the other hand, a similar 50% reduction of the enzymatic activity of MT1-MMP was found with these two downregulated D2A1 cell lines, also quantified in tumor homogenates. These discrepancies highlight that MT1-MMP expression and activity are depended on the tumor microenvironment, and its accurate quantification could be complex.

Our results also showed that only a partial downregulation of the MT1-MMP was required to completely inhibit the development of lung metastases induced by the preirradiated mammary gland. This result suggests the existence of a threshold of MT1-MMP expression above which the risk of early recurrence increases.

MT1-MMP localisation in tumor cells may also have a predictive value. MT1-MMP protein is localised at the membrane and cytoplasm of normal and tumor adjacent tissues (Ip *et al*, 2007). In hepatocellular carcinoma, overexpression of MT1-MMP and its atypical localisation in the nucleus were associated with promotion of metastasis and poor overall survival (Ip *et al*, 2007). In the D2A1-wt and mock tumors, localisation of MT1-MMP in the nucleus was observed. The downregulation of MT1-MMP expression has reduced the accumulation of MT1-MMP in nucleus, but only when the D2A1 cells were implanted in the preirradiated microenvironnement. This reduction of nuclear MT1-MMP was associated with the inhibition of radiation-stimulated metastasis development. The

mechanism involved still need to be determined, but these results suggest that it would be appropriate to determine the tumor cell localisation of MT1-MMP in TNBC biopsies to properly assess its association with the increase of cancer cell invasiveness following radiotherapy rather than its expression only.

Some inflammatory cytokines induced in the irradiated mammary gland could also modify the expression and activity of MT1-MMP (Bouchard *et al*, 2013). Among them, interleukin-6 (IL-6) and IL-1β can directly increase the expression of MT1-MMP and therefore participate in ECM remodelling (Siwik *et al*, 2000; Wilson, 2006; Feng *et al*, 2010; Petrella & Vincenti, 2012). These cytokines were also associated with poor prognosis in breast cancer patients (Zhang & Adachi, 1999; Okamoto *et al*, 2010). Identification of the inflammatory cytokines induced during radiotherapy may contribute to clarify their potential role in radiation-stimulated metastasis development. This information would support the importance of determinating the expression of these cytokines and their receptors in tumor biopsies to better predict the risk of early recurrence before radiation therapy.

MT1-MMP can also affect the cellular metabolism by modulating the activity of HIF-1α. This hypoxic inducible factor plays a key role in the cellular adaptation to hypoxia by inducing the expression of glycolysis-related genes (Sakamoto *et al*, 2014). HIF-1α also regulates angiogenesis by inducing vascular endothelial growth factor (VEGF) expression and promotes cell motility and invasion (Semenza *et al*, 1994; Lee *et al*, 2004). Therefore, hypoxic tumor microenvironment can promote breast cancer invasion (Munoz-Najar *et al*, 2006). During normoxia, HIF-1α is usually strongly suppressed by the factor inhibiting HIF-1 (FIH-1α). However, in certain type of cells including malignant tumor cells, the cytoplasmic tail of MT1-MMP binds to FIH-1α which allows HIF-1α to mediate glycolysis and enhances tumorigenicity (Koziol *et al*, 2012; Sakamoto *et al*, 2014).

In our TNBC model, downregulation of MT1-MMP has resulted in an up-regulation of the HIF-1α pathway as shown by nuclear and cytoplasm mixed expression. A reduction of MT1-MMP increases the availability of FIH-1α that bounds to and inactivates HIF-1α.

Consequently, the production of ATP from glycolysis is not induced, which could contribute to the lower tumor growth rate and increased hypoxia observed for knockdown tumors implanted in irradiated mammary glands. Since HIF-1α also regulates cancer cell invasion, the inactivation of the protein could also support the reduction of radiation-stimulated CTC and lung metastases that has occurred by downregulating MT1-MMP in the D2A1 cells.

In conclusion, our study justifies the initiation of a clinical trial to determine whether the expression of MT1-MMP and its localisation in tumor cells could contribute to the identification of TNBC patients at higher risk of early recurrence.

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Author contributions

GB performed all animal experiments, analyses, result interpretations and drafted the manuscript. HT generated FUCCI cells and performed *in vitro* experiments. GB, CS, RB and BP conceptualized the study. SG contributed to pathological analysis. BP, CS, RB, YBL and SG contributed to writing and revising the manuscript. All authors contributed to critical analysis and approval of the final manuscript.

Disclosure of potential competing financial interests

The authors declare no competing financial interests.

Supplementary material

Supplementary methods

Quantitative Polymerase Chain Reaction (qPCR)

RNA extractions were performed on cell pellets with the Absolutely RNA Microprep Kit (Stratagene) as recommended by the manufacturer, except that DNAse treatments were done at 37°C. Reverse transcription was performed with 2 μg total RNA in a total volume of 20 μL containing Transcriptor reverse transcriptase, random hexamers, deoxyribonucleotides (dNTP) (Roche Diagnostics), and 10 units of RNAseOUT (Invitrogen) following the manufacturer's protocol. All forward and reverse primers were individually resuspended in stock solutions (20–100 μM) containing Tris-HCL buffer and subsequently diluted as a primer pair down to 1 μM in RNase DNase-free water (IDT). qPCR reactions were performed in 10 μL in 96-well plates on a Realplex2 thermocycler (Eppendorf) with 5 μL of 2X FastStart Universal SYBR Green Master mix (Roche Diagnostics), 10 ng (3 μL) cDNA, and 200 nM final (2 μL) primer pair solutions. The following cycling conditions were used: 10 min at 95°C; 50 cycles: 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C. Relative expression levels were calculated using the qBASE framework. The following housekeeping genes were used as internal standard: ubiquitin C (UBC), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde-3-phosphate desidrogenase (GAPDH). In every qPCR run, a no template control was performed for each primer pair and these were consistently negative. Primer sequences: Mmp 14 (MT1-MMP); sense primer 5′-CCCTCGCTGTGGTGTTCCG-3′, antisense primer 5′-TGTGGCATACTCGCCCACCTTA-3′. UBC; sense primer 5′-CGTCGAGCCCAGTGTTACCACCAAGAAGG-3′, antisense primer 5′-CCCCCATCACACCCAAGAACAAGCACAAG-3′. HPRT1; sense primer 5′-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3′, antisense primer 5′-

CCCTGAAGTACTCATTATAGTCAAGGGCAT-3′. GAPDH; sense primer 5′-TGACGTGCCGCCTGGAGAAA-3′, antisense primer 5′-AGTGTAGCCCAAGATGCCCTTCAG-3′.

Western blot

Protein extractions were performed using tumour homogenates in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% NP-40, 0.5% Na-deoxycholate and 5 mM EDTA supplemented with the protein inhibitor cocktail Complete Mini, EDTA-free (Roche Diagnostics). Cellular debris was cleared by centrifugation and supernatants were aliquoted and stored at -80°C for further use. Protein quantification assay was performed with a DC Protein Assay kit (Bio-Rad). The protein extracts were applied on a 12% polyacrylamide-SDS gel at 120 V during 3 hours at 4°C and transferred to a PVDF membrane (Millipore) using the Mini Trans-Blot Cell (Bio-Rad) settled at 100 V for 1 hour. The membrane was blocked with 8% reconstituted skim milk powder in TBST solution (10 mM Tris–HCl pH 7.5 containing 150 mM NaCl and 0.05% Tween 20). The blots were incubated with MT1-MMP antibody (1:750, sc30074, Santa Cruz) in blocking solution overnight at 4°C. After washing with TBST, rabbit horseradish peroxidase-conjugated secondary anti-bodies (1:10 000, LS-C181152, LifeSpan BioSciences) were applied and the blots developed by the Enhanced Chemiluminescence Detection System (Perkin Elmer). Relative intensity of the bands were normalised to α-tubulin internal standard.

Gelatin zymography

MMP-2 levels were analysed by zymography in D2A1 mammary tumours expressing or deficient for MT1-MMP. Briefly, gel zymography is based on a nonreducing acrylamide gel polymerised with gelatin, a substrate of MMP-2. After migration, the gel was incubated under appropriate conditions which allowed metalloproteinases to locally digest the gelatin that appeared as clear bands after staining with Coomassie blue. Tissues were homogenised using 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% NP-40, 0.5% Na-deoxycholate and 5 mM EDTA supplemented with the protein inhibitor cocktail Complete Mini, EDTA-free (Diagnostics). Aliquots of 5 µg were

applied on a 12% polyacrylamide sodium dodecyl sulfate gel containing 0.2% gelatin and electrophoresed at 150 V during 3 hours at 48°C. After removal of SDS from the gel by incubating in 2.5% Triton X-100 (30 min, room temperature), the gel was incubated at 37°C for 18 hours in 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$,1 mM $ZnCl_2$, 1% Triton, and stained with Coomassie blue R-250. Four independent assays were performed.

Immunohistochemistry (IHC)

IHC were performed on D2A1 FUCCI tumour frozen sections for the detection of the CD31 blood vessel marker (sc-1506; 7 µm; dilution 1:100; Santa Cruz Biotechnology), MT1-MMP (sc-30074; 7 µm; dilution 1:100; Santa Cruz Biotechnology), HIF-1α hypoxia marker (NB-100-654; 7 µm; dilution 1:250; Novus Biologicals) and Ki67 proliferation marker (ab15580; 3 µm; dilution 1:100; Abcam Inc.) using previously described procedure[1]. MT1-MMP in tumour-free mammary glands were detected on paraffin-embedded sections. Sections were deparaffinised in xylene and boiled in citrate buffer (pH 6) for 5 minutes using a pressure cooker. Peroxydases were blocked with 3% H_2O_2 for 10 minutes and washed with PBS. Tissues were then blocked with BSA 10% for 1 hour at room temperature and incubated over night with MT1-MMP antibody (sc30074; 5 µm; dilution 1:100; Santa Cruz Biotechnology) at 4°C. Signal revelation was realised using an HRPO secondary antibody and the Dako EnVision HRP system. Tissues were counterstained with methyl-green.

Results were expressed as percentage of CD31 stained area in the field, percentage of Ki67 positive cells or intensity/localisation of MT1-MMP and HIF-1α staining. Nuclei were quantified using ImageJ image-based tool for counting nuclei plug in. Staining intensity was measured according to Pham *et al.* method[2] adapted by **Plateforme d'Analyse et de Visualisation d'Images** (PAVI) of the Université de Sherbrooke.

For each tissue, images of 5 to 10 representative areas were taken (magnification X 200 or 400) for quantification.

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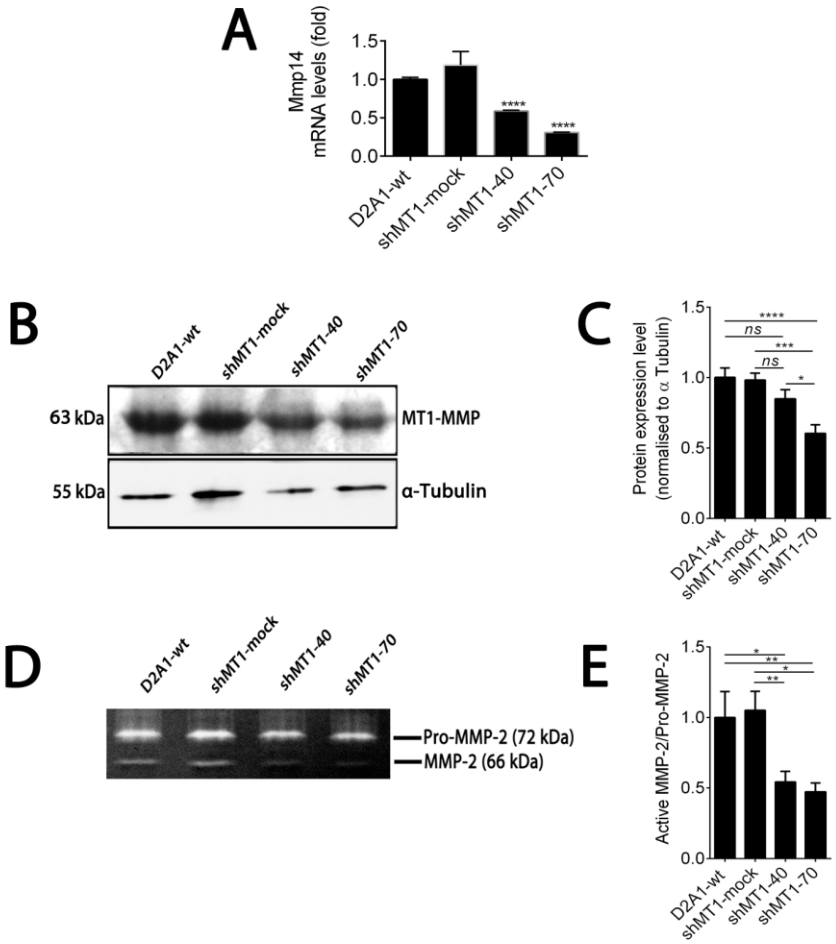
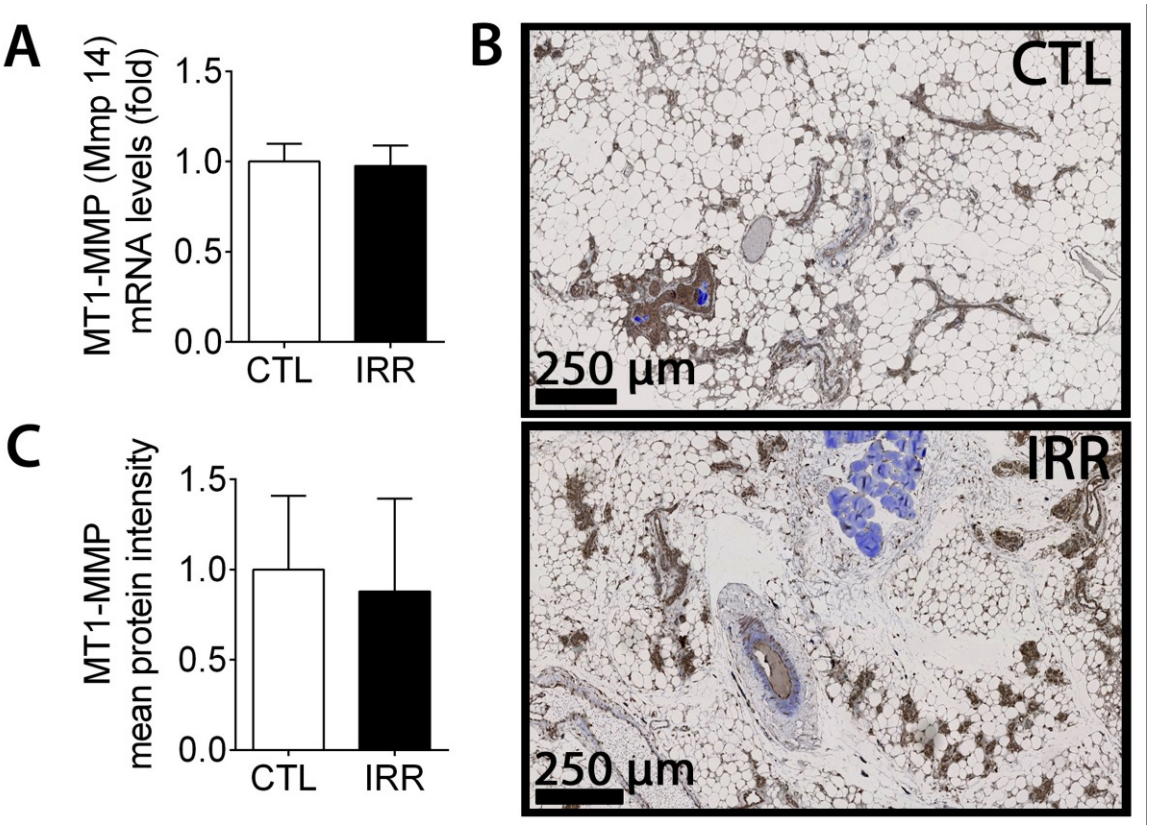
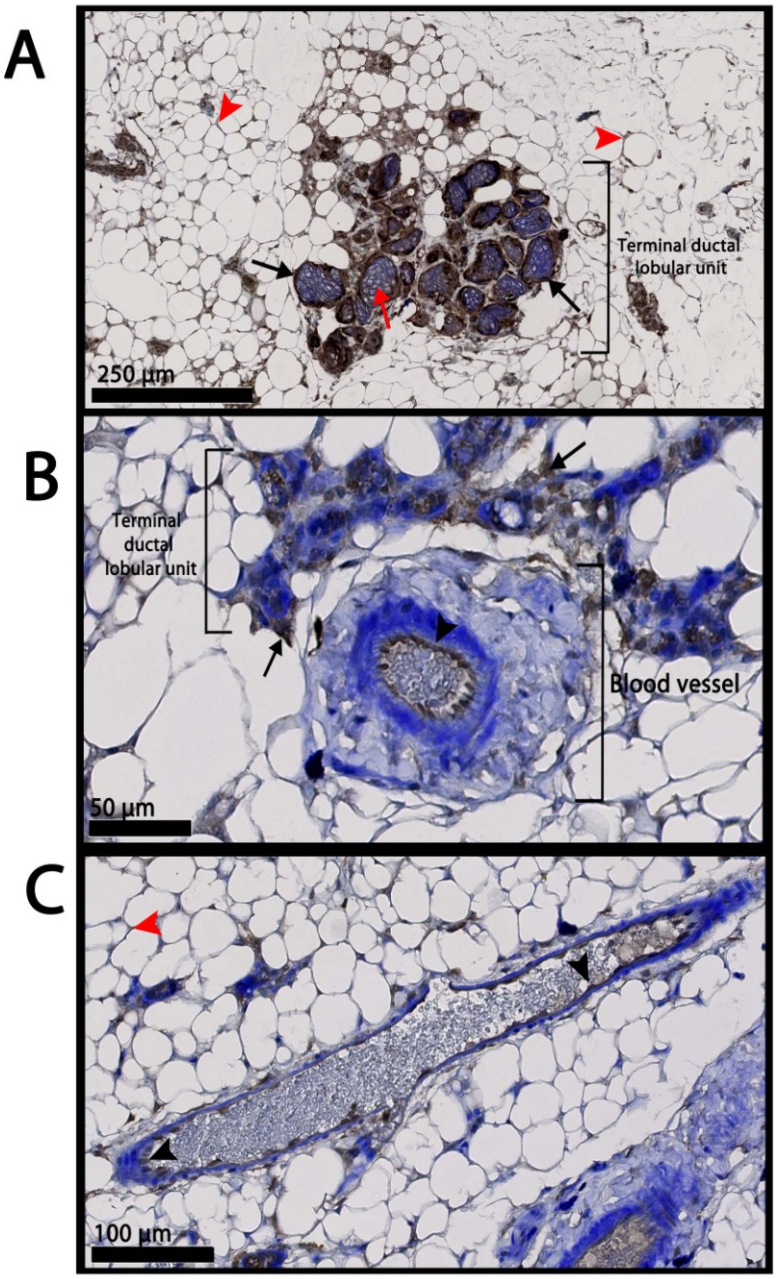
Supplementary figures



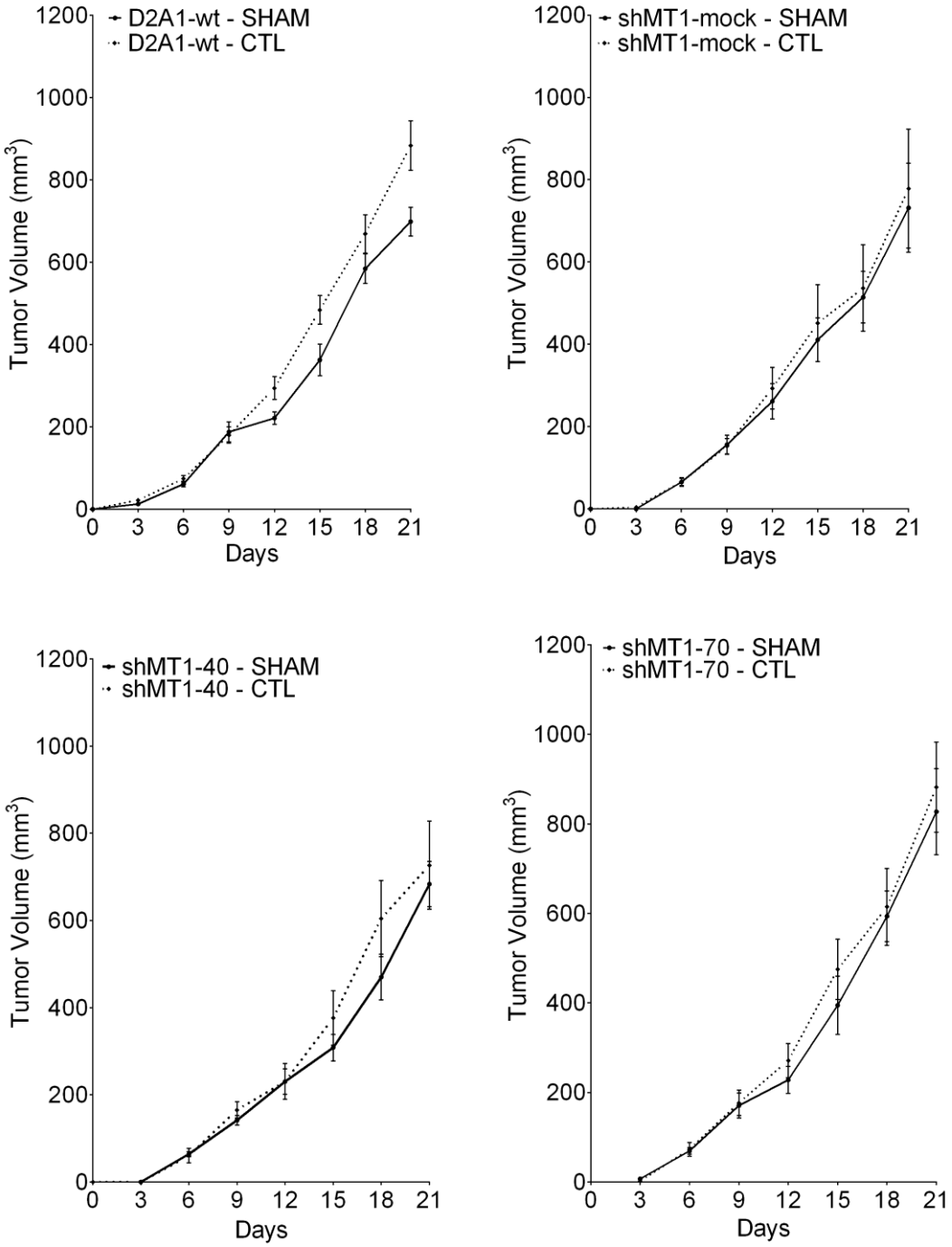
Supplementary Figure S1. MT1-MMP downregulation with shRNA. **(A)** qPCR showing no reduction of the mRNA transcript in the mock cell line, a diminution of 40 % ($P < 0.0001$) in shMT1-40 cell line and a diminution of 70 % ($P < 0.0001$) of the mRNA transcript in shMT1-70 cell line compared to the D2A1- wt (n = 3). **(B)** Western blot showing a decrease of the MT1-MMP protein expression. **(C)** Western blot quantification of the MT1-MMP band. The MT1-MMP protein expression was significantly reduced in shMT1-70 tumour ($P < 0.0001$), but not in the shMT1-40 tumour ($P = 0.0977$) (n = 9). **(D)** Zymogram gel showing a diminution of the activation of proMMP-2 to MMP-2, the main substrate of MT1-MMP. **(E)** Zymography quantification of the pro- and active MMP-2 bands. The enzymatic activity of MT1-MMP was significantly reduced in shMT1-40 ($P = 0.0149$) and shMT1-70 cell lines ($P = 0.0018$) (n = 8 to 14).



Supplementary Figure S2. MT1-MMP expression in tumour-free mammary gland 6 hours after the last radiation fraction. **(A)** Mmp 14 mRNA transcripts (MT1-MMP) were quantified by qPCR (n = 3). No radiation-enhancement was observed. **(B)** Quantification of IHC MT1-MMP staining performed on control and irradiated paraffin-embedded mammary glands sections. No radiation-enhancement was observed (n = 3). **(C)** Representative IHC images after MT1-MMP staining in control and irradiated mammary glands. CTL = control, IRR = irradiated.



Supplementary Figure S3. MT1-MMP positive controls supporting the specificity of the antibody sc-30074 performed on normal paraffin-embedded mammary glands sections. **(A)** IHC against MT1-MMP showing positive staining for myoepithelial cells (black arrows) and light staining in adipocytes (red arrowheads) as well as negative staining for epithelial cells of a terminal ductal lobular unit of breast tissue (red arrows). **(B)** IHC against MT1-MMP showing positive staining for myoepithelial cells around a terminal ductal lobular unit (black arrows) and endothelial cells of blood vessels (black arrowhead) as well as negative staining for other blood vessel components. **(C)** IHC against MT1-MMP also showing positive staining for endothelial cells of a blood vessel (black arrowheads) and light staining in adipocytes (red arrowhead).



Supplementary Figure S4. Validation that tumour growth was not affected by systemic factors released by irradiating the mammary glands. D2A1 tumour volumes of sham irradiated animals (sham tumours) were comparable to those implanted in the left nonirradiated mammary gland of mice whose third right mammary gland was preirradiated. (n = 10 to 36).

DISCUSSION

La radiothérapie est sans aucun doute un traitement très important dans la guérison du cancer du sein. Par contre, de plus en plus d'études, ainsi que cette thèse, appuient le potentiel de la radiation à augmenter l'invasion des cellules cancéreuses du sein, et ce, principalement chez certains sous-groupes de patientes. Toutes les patientes sont actuellement traitées avec le même régime de RT sans savoir si celles-ci sont à risque de voir leur cancer progresser suite au traitement. De ce fait, il est impératif d'identifier ces patientes. Le but de cette thèse présentait deux objectifs principaux:

- Mettre en évidence et mieux comprendre les phénomènes de migration et d'invasion radio-induite chez les TNBC.
- Identifier un biomarqueur de prédiction des effets de la radiation afin d'anticiper et optimiser la RT chez les TNBC.

Cette thèse a confirmé le rôle de l'inflammation dans l'invasion radio-induite. Plus précisément, la voie de signalisation IL-1β/COX-2/prostaglandines/MMPs a été identifiée dans chacun des modèles expérimentaux, appuyant fortement son importance dans ce phénomène. De plus, cette étude suggère que l'expression ainsi que la localisation de la MT1-MMP dans les cellules cancéreuses en font un biomarqueur de choix dans la prévention de l'invasion radio-induite.

1. DISCUSSION DES ARTICLES

OBJECTIF #1

Article 1: L'induction de l'interleukine 1β par l'irradiation d'une tumeur mammaire de souris de cancer du sein triple négatif favorise l'invasion et la formation de métastases

Le rôle premier du traitement de RT est d'éliminer les cellules cancéreuses résiduelles dans les marges chirurgicales. Ce traitement est normalement administré après la résection de la tumeur primaire, mais il arrive dans certains cas plus rares de cancer du sein que la RT soit utilisée comme thérapie pré-opératoire. Par exemple, la RT néoadjuvante peut être utilisée lorsque la chimiothérapie n'a pas suffisamment réduit la tumeur pour permettre une résection en marges négatives. De ce fait, il est impératif de bien comprendre l'effet de la RT, autant sur les cellules cancéreuses que sur leur microenvironnement. Cet article avait pour but d'établir un profil d'expression cytokines inflammatoires pendant le traitement de RT pour ainsi déterminer les facteurs impliqués dans l'invasion radio-induite chez les TNBC. En résumé, ce modèle murin de TNBC consistait à irradier une tumeur D2A1 une semaine post-implantation afin de quantifier différentes cytokines pro-invasives dans le plasma sanguin avant, pendant et après le traitement de RT. Cette étude visait aussi à démontrer que la RT peut faire progresser une tumeur en augmentant les CTC et les métastases pulmonaires.

Des 6 cytokines pro-inflammatoires quantifiées dans cette étude, une seule a été significativement augmentée dans le plasma sanguin pendant le traitement de RT, soit l'IL-1β. Cette cytokine est exprimée par la cellule tumorale (Lewis *et al.*, 2006) ou encore par les cellules stromales (Paquette *et al.*, 2013a) puis est activée par l'enzyme de conversion de l'interleukine (ICE). Cette forme mature est ensuite sécrétée et stimule les cellules du microenvironnement de manière autocrine ou encore paracrine. L'IL-1β est bien connue pour ses rôles autant physiologiques comme entre autres dans la perméabilité vasculaire ou encore pathologiques comme son implication dans la progression tumorale. Par exemple, l'IL-1β augmente l'expression de plusieurs gènes pro-métastatiques comme les MMPs, le VEGF ou encore le TGF-β. Plusieurs patients atteints d'arthrite rhumatoïde ou encore de cancer ont été rapportés pour avoir des concentrations élevées d'IL-1β dans la circulation sanguine ou au sein de la tumeur généralement associées avec un mauvais pronostic de cancer (Lewis *et al.*, 2006).

Bien que l'IL-1β soit connue pour son rôle potentiel dans l'invasion du cancer, le mécanisme d'induction des métastases associé n'est pas clairement défini. Le groupe de Paquette *et al.* a démontré l'importance de d'IL-1β dans un modèle *in vitro* d'invasion radio-induite des cellules cancéreuses du sein MDA-MB-231 (Paquette *et al.*, 2013a). Dans ce

modèle, les cellules se sont avérées significativement plus agressives lorsque stimulées par de l'IL-1β libérée par des fibroblastes irradiés. L'IL-1β a été associée à ce phénomène en raison de l'expression de son ARNm significativement augmentée ainsi qu'une induction de la voie des prostaglandines, connue pour être stimulée par cette cytokine. Une expérience supplémentaire pour valider cette hypothèse a été réalisée en stimulant directement les MDA-MB-231 avec de l'IL-1β. Comme attendu, leur invasion a été significativement augmentée. Dans le même ordre d'idées, l'ajout d'un anticorps contre cette cytokine inhiba complètement l'invasion induite par le microenvironnement irradié dans ce même modèle. En comparaison, les résultats du premier article présenté dans cette thèse démontrent que l'irradiation des cellules cancéreuses de souris triple négatives D2A1 diminue significativement leur invasion *in vitro*. Par contre, un ajout d'IL-1β dans le milieu environnant renverse complètement ce processus et réinstaure l'état agressif initial des cellules cancéreuses. Ce résultat confirme les résultats précédents du rôle de IL-1β dans l'invasion des cellules cancéreuses et nous porte à croire que cette cytokine pourrait jouer un rôle clé dans l'invasion radio-induite. En lien, il n'est pas surprenant que l'IL-1β soit aussi augmentée dans le modèle *in vivo* d'irradiation présenté dans le premier article de cette thèse. Ce modèle animal de TNBC a permis de mettre en évidence une augmentation du nombre de CTC dans la circulation sanguine simultanément avec l'augmentation d'IL-1β plasmatique pendant le traitement de RT, menant par la suite à une augmentation du nombre de métastases pulmonaires chez les souris irradiées. Il est aussi intéressant de mentionner que l'IL-1β et le nombre de CTC sont rapidement revenus au même taux basal que les souris non-irradiées, et ce, aussi tôt que 7 jours post-irradiation. Cette étude confirme donc l'importance de l'IL-1β dans l'initiation du phénomène d'invasion radio-induite, mais remet en question la quantification des CTC dans la circulation sanguine pour estimer le nombre de métastases pulmonaires puisque les CTC chez les souris irradiées reviennent rapidement au même taux que chez les souris non-irradiées. Ce résultat démontre que dépendamment du moment où les CTC sont quantifiées, ce nombre ne reflète pas nécessairement le profil métastatique. Aussi, bien que le nombre de CTC ait été augmenté par l'irradiation, une certaine partie de ces cellules est probablement attribuable à une dégradation mécanique de la tumeur causée par la RT. D'autre part, il est évident que certaines de ces cellules étaient toujours viables dans la circulation sanguine, car une

augmentation significative du nombre de métastases pulmonaires a été observée chez les souris irradiées à la tumeur. L'hypothèse que la RT induit des CTC viables dans la circulation sanguine a d'ailleurs été confirmée par le groupe de Martin *et al.* dans des échantillons sanguins de patients atteints de cancer du poumon non à petite cellules (Martin *et al.*, 2014).

Manifestement, l'IL-1β est une cytokine importante dans la progression du cancer. Une revue exhaustive sur le potentiel des inhibiteurs de l'IL-1β ou de son récepteur dans le traitement du cancer a été publiée dans le *Journal of Transnational Medicine* par le groupe de Lewis *et al.*. En lien, des antagonistes du récepteurs de l'IL-1β ou encore des anticorps monoclonaux contre celui-ci sont déjà utilisés dans le traitement de l'arthrite rhumatoïde. Des études pré-cliniques ont aussi démontré le potentiel des anticorps monoclonaux à ralentir la croissance tumorale, le taux de prolifération cellulaire, la libération de facteurs angiogéniques et même de diminuer les métastases (Lewis *et al.*, 2006). L'utilisation de ces inhibiteurs conjointement avec les traitement actuels contre le cancer semblent très prometteurs. De ce fait, notre équipe de recherche travaille actuellement à la mise en place d'un projet pilote clinique visant à quantifier l'IL-1β dans le plasma de patientes atteintes de TNBC afin d'identifier celles qui seraient le plus à risque de récidive précoce reliée au phénomène d'invasion radio-induite.

Bien que ce modèle met l'emphase sur l'augmentation de la migration des cellules cancéreuses stimulées par l'IL-1β radio-induite, il ne faut pas omettre le potentiel des radiations à occasionner des mutations génétiques. Comme décrit dans l'introduction de cette thèse, ces mutations radio-induites peuvent favoriser un phénotype agressif, favorisant aussi l'invasion de la cellule. Des expériences supplémentaires seraient nécessaires afin de confirmer cette hypothèse dans le modèle d'étude de ce premier article.

Article 2: La pré-irradiation de la glande mammaire de souris stimule la migration des cellules cancéreuses du sein et le développement des métastases pulmonaires

Le premier article de cette thèse a montré que la migration des cellules cancéreuses est augmentée dans la glande mammaire de la souris post-irradiation de la tumeur menant à

une augmentation des CTC et du nombre de métastases pulmonaires. Comme mentionné plus haut, il est très rare dans la pratique clinique qu'une tumeur du sein soit irradiée avant sa résection chirurgical. Ainsi, l'irradiation cible principalement le tissu mammaire sain, justifiant la pertinence d'élaborer un modèle de pré-irradiation de la glande mammaire avant l'implantation des cellules cancéreuses. Ce modèle animal a permis de mettre en évidence le potentiel métastatique du stroma irradié indépendamment des cellules cancéreuses irradiées.

En bref, ce modèle animal consistait à irradier la glande mammaire droite de la souris Balb/c avec 4 fractions de 6 Gy à 24 h d'intervalle pour ensuite y injecter les cellules de carcinome du sein D2A1 des deux côtés dans la troisième paire de glande mammaires. Cet article met en évidence l'influence du stroma irradié sur l'agressivité des cellules cancéreuses. En effet, dès les premières mesures des courbes de croissances tumorales, les tumeurs implantées dans les glandes mammaires pré-irradiées semblaient déjà beaucoup plus petites. Ce volume réduit s'explique par le fait que les cellules tumorales s'infiltrent plus rapidement dans le tissu, migrant plus loin et plus rapidement, menant à une masse tumorale plus petite en comparaison des tumeurs implantées du côté contrôle. En lien avec cette augmentation de la migration mesurée par imagerie optique et confirmée histologiquement, un nombre plus élevé de CTC dans le sang ainsi que de métastases pulmonaires a été observé chez les souris pré-irradiées à la glande mammaire. L'hypothèse de dommages vasculaires radio-induits facilitant l'intravasation des cellules cancéreuses dans la circulation a été exclue car les coupes histologiques des glandes mammaires irradiées n'en démontrent aucun signe (Figure 1).

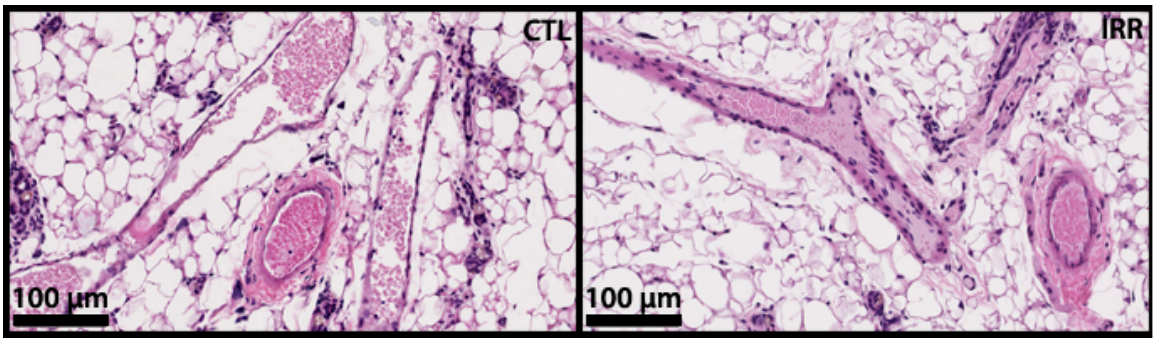


Figure 1. **Intégrité vasculaire de la glande mammaire de souris post-irradiation.** Coloration H&E de glandes mammaires de souris contrôle (CTL) et irradiée (IRR) illustrant des vaisseaux sanguins. Aucun dommage significatif ne semble avoir affecté la paroi vasculaire des glandes mammaires irradiées avec 4 fractions de 6 Gy à 24 h d'intervalle en comparaison aux vaisseaux contrôles.

Suite aux conclusions du premier article, l'inflammation radio-induite a pu être envisagée comme étant une hypothèse plausible pour expliquer l'augmentation du nombre de métastases pulmonaires. De ce fait, plusieurs molécules inflammatoires ont été quantifiées dans la glande mammaire irradiée 6h après la dernière séance d'irradiation fractionnée. L'IL-6 ainsi que la voie des prostaglandines, supportée par une augmentation significative de la COX-2, la PLA2 et la PGE2, se sont avérées significativement augmentées par l'irradiation. Étonnamment, aucune augmentation significative de l'IL-1β n'a été observée dans la glande mammaire irradiée alors que cette cytokine semble jouer un rôle clé dans l'invasion radio-induite selon les résultats de plusieurs études, incluant les résultats du précédent article présenté dans cette thèse (Desmarais *et al.*, 2012; Paquette *et al.*, 2013a). Plus précisément, un modèle de pré-irradiation du cerveau sain avant l'implantation des cellules de glioblastome F98 démontre des résultats très semblables à ceux observés dans cette étude (Desmarais *et al.*, 2012). Une augmentation significative de la migration des cellules cancéreuses dans le parenchyme irradié a été observée au détriment du volume de la tumeur primaire. Ultimement, l'augmentation de l'agressivité des cellules induites par le parenchyme irradié a diminué significativement la survie des rats dans cette étude. En lien, l'inflammation radio-induite médiée par la voie des prostaglandines a aussi été proposée comme joueur important dans ce phénomène. Il est intéressant de noter que dans cette étude, l'expression la plus élevée d'IL-1β a été quantifiée 4 h post-irradiation en comparaison à 6 h post-irradiation dans notre étude. Selon le modèle

proposé par Paquette *et al.* (Paquette *et al.*, 2013b), l'IL-1β serait en amont de la cascade de signalisation de l'invasion radio-induire. Ce résultat nous laisse supposer que le moment de quantification de l'IL-1β dans notre étude n'était probablement pas adéquat et qu'un temps plus tôt post-irradiation aurait probablement permis d'observer une augmentation significative.

Cet article supporte aussi l'hypothèse que la provenance des MMPs origine des cellules malignes irradiées plutôt que du stroma irradié. Utilisant le même modèle animal ainsi que les même cellules de cancer du sein que l'article précédent présenté dans cette thèse, aucune augmentation des MMPs n'a été observée dans de la glande mammaire irradiée pas plus que dans les homogénats de tumeurs (cellules cancéreuses D2A1 + glande mammaire pré-irradiée) dans ce modèle de pré-irradiation de la glande mammaire. En comparaison avec l'article précédent, l'irradiation de la tumeur a provoqué une augmentation de la MMP-2 et -9 supportant ainsi l'augmentation de l'expression des MMPs par les cellules cancéreuses plutôt que par le stroma irradié.

Bien que l'idée que l'irradiation puisse faire progresser ou augmenter le potentiel métastatique de certains cancers soit toujours controversée, d'autres modèles *in vivo* ainsi que cet article appuient fermement cette idée. Entre autres, l'équipe de Barcellos-Hoff a largement étudié les effets de la glande mammaire irradiée et est très bien connue comme étant un groupe de pionniers sur le sujet par la communauté scientifique. D'ailleurs, un article de ce même groupe publié dans le journal *Cell* décrit un modèle murin d'irradiation du corps entier dans lequel on réimplante des cellules de glande mammaire saine provenant du même hôte qui avaient été préalablement retirées et mises en culture (Nguyen *et al.*, 2011). Le développement de tumeurs spontanées avec un phénotype plus agressif de type estrogène-négatif a été observé chez les souris irradiées réimplantées avec ces cellules en comparaison avec les quelques tumeurs observées chez les souris non-irradiées, généralement de plus petites tailles et de phénotype moins agressif. Ces résultats vont dans le même sens que nos observations en appuyant le potentiel du stroma irradié à promouvoir l'agressivité des cellules cancéreuses du sein.

Ces deux premiers articles présentés dans cette thèse démontrent le potentiel de l'irradiation à promouvoir la migration et l'invasion des cellules D2A1 dans un modèle de cancer TNBC chez la souris. Autant l'irradiation de la tumeur que l'irradiation de la glande

mammaire ont provoqué une augmentation de la migration des cellules cancéreuses dans le parenchyme et une augmentation des CTC menant ultimement à une augmentation du nombre de métastases pulmonaires. Ces deux modèles ont aussi mis en évidence l'importance de l'inflammation radio-induite dans ce phénomène. En lien avec ces résultats, un traitement anti-inflammatoire administré conjointement avec la RT pourrait prévenir le phénomène d'invasion radio-induite et potentiellement augmenter la survie chez les patientes atteintes de TNBC.

Article 3: Prévention de la migration et de la formation de métastases pulmonaires par la chloroquine dans un modèle de souris pré-irradiée à la glande mammaire

Ce troisième article avait pour but de prévenir la migration radio-induite menant à une augmentation des métastases pulmonaires tel qu'observé dans les deux modèles *in vivo* précédents. L'hypothèse principale de cet article est basée sur le principe que cet effet néfaste des radiations pourrait être prévenu en inhibant l'inflammation radio-induite. Pour cette étude, le même modèle de pré-irradiation de la glande mammaire a été utilisé, mais cette fois-ci les souris ont été traitées à la CQ avant chaque dose d'irradiation puis pendant la croissance tumorale. La CQ est un agent anti-malarien aussi utilisé comme anti-inflammatoire large spectre dans le traitement de maladies auto-immunes comme l'arthrite rhumatoïde ou le lupus érythémateux (Solomon and Lee, 2009). Dans cette étude, l'action préventive de la CQ contre l'augmentation de la migration des cellules cancéreuses, des CTC ainsi que du nombre métastases pulmonaires radio-induites est décrite pour une première fois.

Les résultats *in vitro* de cette étude démontrent un effet très intéressant. Dans un modèle de coculture en chambre d'invasion, les fibroblastes irradiés ont su augmenter significativement l'invasion de plusieurs lignées cancéreuses du sein autant humaines que murines, mais étonnamment, seulement les cellules triple négatives ont été stimulées par le microenvironnement irradié. Aussi, le traitement des cellules avec la CQ a complètement inhibé cet effet invasif sans impacter leur taux basal d'invasion car le même nombre de

cellules cancéreuses a traversé la membrane basale avec ou sans traitement à la CQ. Cela supporte l'idée que la CQ inhibe sélectivement l'invasion induite par la RT, et ce principalement chez les cellules de TNBC puisque les autres lignées cellulaires de cancer du sein estrogène-positives utilisées dans ce modèle n'ont montré aucune augmentation.

Chez les souris traitées à la CQ cette fois-ci, cette étude a démontré une réduction significative de la croissance tumorale, une diminution de la migration des cellules dans le parenchyme mammaire irradié et une inhibition des CTC ainsi que des métastases pulmonaires radio-induites. Comme espéré, la CQ a diminué significativement l'expression de plusieurs gènes associés à l'inflammation. Entre autres, une diminution de l'IL-1β ainsi que l'IL-6, bien connues pour induire l'invasion des cellules cancéreuses (Lewis *et al.*, 2006; Nicolini *et al.*, 2006; Dethlefsen *et al.*, 2013), a été observée dans les glandes mammaires contrôles et irradiées. Un autre résultat intéressant observé dans cette étude concerne l'effet de la CQ en lien avec la COX-2. Étant donné que la COX-2 est induite seulement en cas d'inflammation ou de pathologie, l'effet inhibiteur de la CQ affecte sélectivement l'induction radio-induite de la voie des prostaglandines et n'a aucun effet sur les glandes mammaires contrôles. Ce résultat est très attrayant du côté de la pratique clinique, car il démontre le potentiel d'un inhibiteur sélectif de cette voie à préserver l'intégrité des tissus sains en ciblant exclusivement le phénomène d'invasion radio-induit médié par la COX-2. Appuyant cette affirmation, une étude précédente *in vitro* effectuée au laboratoire a démontré qu'un inhibiteur sélectif à la COX-2, le NS-398, peut inhiber ce phénomène chez les cellules humaines de cancer du sein triple de négatif MDA-MB-231 (Paquette *et al.*, 2013b). Encore dans notre laboratoire, l'inhibition de l'invasion radio-induite en utilisant des inhibiteurs sélectifs de la COX-2 a aussi été démontrée dans un modèle de cerveau de rat irradié (Desmarais *et al.*, 2015) ou encore chez la souris dans un modèle de pré-irradiation du muscle de la cuisse (Lemay *et al.*, 2015, soumis). Dans ces deux modèles, l'invasion des cellules cancéreuses stimulées par l'irradiation, que celle-ci soit administrée en dose unique ou fractionnée, a été prévenue avec succès.

Bien que les résultats de ce troisième article aillent dans le même sens que les autres études utilisant des inhibiteurs sélectifs de la COX-2 réalisées dans notre laboratoire, il nous est impossible d'émettre cette même conclusion hors de tout doute pour cette étude puisque la CQ n'est pas une drogue spécifique à la COX-2. La faiblesse principale de cette

étude est donc en lien avec l'effet lysosomotropique de la CQ qui affecte des dizaines d'enzymes impliquées dans maintes voies de signalisation pouvant ultimement causer des effets secondaires même si aucune toxicité notable n'a été remarquée chez les souris dans cette étude. Le manque de sélectivité de cette drogue ne nous permet donc pas d'interpréter les résultats avec précision, mais seulement d'émettre une hypothèse générale. Des études supplémentaires utilisant un inhibiteur sélectif à la COX-2 ou un autre inhibiteur sélectif affectant cette même voie de signalisation seront nécessaires pour confirmer que l'invasion radio-induite est COX-2-dépendante dans la glande mammaire de souris.

En bref, l'invasion radio-induite pourrait jouer un rôle clé dans la récidive rapide chez les TNBC. En effet, puisque la majorité de ces patientes sont diagnostiquées avec un phénotype de cancer de plus haut grade histologique, un traitement de RT est donc administré dans la quasi-majorité des cas, laissant supposer que l'invasion radio-induite pourrait être une cause de récidive précoce pour un sous-groupe de ces patientes. Cependant, il est actuellement impossible de valider cette hypothèse avec les banques de données publiques (ex. *The Cancer Genome Atlas*) puisque le nombre de cas de patientes TNBC irradiées ayant subi une récidive précoce avec un suivi clinique complet et bien documenté associé est minime. Selon les résultats *in vitro* et *in vivo* provenant de ce troisième article, la CQ semble être un traitement intéressant pour prévenir cet effet néfaste des radiations, mais un traitement plus sélectif serait certes une option envisageable pour limiter les effets secondaires possibles liés à la non-spécificité de cette drogue.

OBJECTIF #2

Article 4: La MT1-MMP joue un rôle clé dans le développement de métastases pulmonaires radio-induites dans un modèle de cancer du sein triple négatif chez la souris

Nos résultats précédents ont montré que la CQ prévient l'invasion radio-induite des cellules cancéreuses triple négatives D2A1 dans un modèle de pré-irradiation de la glande

mammaire de souris. Bien que l'hypothèse générale du mécanisme d'action semble impliquer l'inflammation radio-induite COX-2-dépendante, celui-ci n'a pas été confirmé dans ce modèle en raison du manque de spécificité de la CQ.

Une étude antérieure réalisée au laboratoire à mis en évidence le rôle de la MT1-MMP dans cette même voie de signalisation. La MT1-MMP est une métalloprotéinase de matrice ancrée à la membrane cellulaire très bien connue pour son rôle dans la progression tumorale (Seiki, 2003). Plus précisément, la COX-2 induit la production de prostaglandines comme la PGE2, qui à son tour stimule la production de certaines MMPs, notamment la MMP-2. Sachant que le principal activateur de la MMP-2 est la MT1-MMP et que l'utilisation d'un anti-MT1-MMP a su bloquer l'invasion radio-induite des MDA-MB-231 dans un modèle *in vitro* de chambre d'invasion, la MT1-MMP semble être une candidate potentielle intéressante dans le mécanisme d'invasion radio-induite. De ce fait, l'expression de la MT1-MMP a été réprimée à différents niveaux chez les cellules triple négatives de souris D2A1 pour ainsi confirmer son rôle potentiel dans l'invasion radio-induite du TNBC et mieux comprendre son mécanisme d'action. Le quatrième et dernier article de cette thèse met en évidence le rôle de la MT1-MMP dans notre modèle de pré-irradiation du stroma mammaire utilisant cette fois-ci les cellules D2A1 réprimées pour l'expression de cette protéase.

Étonnamment, le niveau de MT1-MMP n'a aucunement affecté la croissance des tumeurs D2A1 implantées dans la glande mammaire non-irradiée. À l'opposé, lorsqu'implantées dans la glande mammaire pré-irradiée, les cellules réprimées pour la MT1-MMP (shMT1-40 et shMT1-70) démontrèrent une croissance tumorale significativement ralentie en comparaison avec les cellules non-modifiées (shMT1-wt et shMT1-mock). En lien, la migration des cellules cancéreuses dans le parenchyme mammaire a été diminuée ainsi que le nombre de CTC et métastases pulmonaires associées chez les souris implantées avec les cellules réprimées pour la MT1-MMP dans la glande mammaire pré-irradiée. Il est aussi intéressant de mentionner que la répression de la MT1-MMP n'a pas diminué le niveau basal de CTC ainsi que le nombre de métastases pulmonaires lorsqu'implantées dans la glande mammaire non-irradiée. Ces résultats supportent le rôle de la MT1-MMP dans l'invasion radio-induite des cellules cancéreuses triple négatives D2A1. Appuyant nos résultats, un article de Perentes *et al.* publié dans

Cancer Research a aussi démontré l'absence d'effet direct de la répression de la MT1-MMP sur la croissance de tumeurs triple négatives chez la souris (Perentes *et al.*, 2011). En comparaison, leur groupe a démontré une diminution du nombre de métastases pulmonaires spontanées alors qu'aucune différence significative n'a été observée dans notre étude. Pourtant, nos résultats *in vitro* semblaient présager que les cellules D2A1 réprimées pour la MT1-MMP démontreraient ce même effet *in vivo,* car leur invasion a été significativement diminuée lors des essais en chambres d'invasion. Cette étude présente par contre des limites au niveau de la répression du gène de la MT1-MMP. Bien que des lignées exprimant 40% et 70% d'ARNm de MT1-MMP aient été créées à partir des D2A1, l'activité catalytique résultante, mesurée par l'activation de la MMP-2, est très semblable pour les deux lignées cellulaires. Ce résultat pourrait donc supporter les résultats comparables obtenus pour les deux lignées réprimées. Il faut aussi prendre en considération que ces deux modèles ne sont pas entièrement équivalents. L'étude de Perentes *et al.* a été effectuée avec les cellules humaines MDA-MB-231 utilisant des souris immunodéficientes de type combinée sévère afin d'éviter un rejet de l'hôte. Ma thèse met en évidence l'importance de l'inflammation dans le phénomène d'invasion radio-induite donc l'utilisation d'un modèle murin est certes moins représentatif de l'humain mais nettement plus approprié pour l'étude d'une telle réaction majoritairement enclenchée par le système immunitaire.

Bien que l'expression de la MT1-MMP ait maintes fois été corrélée avec la progression et un mauvais pronostic de cancer (Perentes *et al.*, 2011; He *et al.*, 2013; Li *et al.*, 2015), une expression de 40% ou 70% d'ARNm en comparaison avec les cellules D2A1 non-réprimées pour la MT1-MMP n'a montré aucune différence notable entre la croissance tumorale, le nombre de CTC ainsi que les métastases pulmonaires radio-induites chez les souris contrôles dans notre étude. Par contre, les coupes histologiques ont fait ressortir une localisation différente de la protéine lorsque les cellules réprimées partiellement pour la MT1-MMP sont implantées dans le tissu pré-irradié. En lien, une étude de Ip *et al.* a montré une localisation atypique de la MT1-MMP au noyau de cellules de carcinomes hépatocellulaires. Cette localisation a été associée à un phénotype tumoral plus agressif et un mauvais pronostic de survie (Ip *et al.*, 2007). Il n'est donc pas surprenant d'observer la MT1-MMP localisée au noyau dans notre modèle d'étude étant donné que les cellules D2A1 sont intrinsèquement de nature très agressive. Par contre, il est très étonnant de

constater que la majorité des noyaux deviennent négatifs pour la MT1-MMP lorsque les cellules réprimées sont implantées dans les glandes mammaires pré-irradiées. Bien que le mécanisme de délocalisation précis de la protéase n'ait pas été identifié dans cette étude, ce résultat montre que la MT1-MMP est nécessaire au phénomène d'invasion radio-induite et qu'une diminution de l'expression de la protéase en contexte de microenvironnement irradié altère l'agressivité des cellules.

En comparaison avec les études précédentes appuyant le rôle de la MT1-MMP dans la progression tumorale, notre étude illustre pour la première fois une cause potentielle de récidive précoce liée avec la RT chez les patientes atteintes de TNBC. Notre étude suggère aussi que la localisation de la MT1-MMP au noyau représente un intérêt clinique notable dans l'identification des patientes à haut risque de récidive radio-induite. En quantifiant l'expression et la localisation de cette protéase, il serait possible d'anticiper une réponse favorable ou non au traitement de radiothérapie. Bien sûr, des études supplémentaires sont nécessaires afin de savoir s'il vaudrait mieux augmenter la dose totale de RT chez les patientes à haut risque de migration radio-induite ou plutôt omettre le traitement.

En lien avec cette découverte, une étude pilote est actuellement en cours dans notre laboratoire. Des biopsies de TNBC provenant de patientes ayant subi de la RT seront analysées pour l'expression de la MT1-MMP ainsi que sa localisation. Nous espérons observer une corrélation avec les femmes qui ont montré une récidive rapide.

2. DISCUSSION GÉNÉRALE.

Les articles présentés dans cette thèse ont su confirmer avec succès les deux hypothèses principales de cette étude à savoir de mettre en évidence et mieux comprendre les phénomènes de migration et d'invasion radio-induite chez les TNBC ainsi que d'identifier un biomarqueur de prédiction des effets de la radiation afin d'anticiper et optimiser la RT chez les TNBC. En premier lieu, les trois premiers articles ont permis de mettre en évidence le phénomène d'invasion radio-induite dans deux différents modèles *in vivo*. Le premier modèle consistait à l'irradiation directe d'une tumeur primaire alors que le deuxième consistait à un modèle de pré-irradiation du stroma avant l'implantation des

cellules cancéreuses du sein. Ces deux modèles ont réussi de manière indépendante à mettre en évidence le potentiel de l'irradiation fractionnée à augmenter la migration des cellules cancéreuses D2A1, les CTC, ainsi que les métastases pulmonaires. Autant l'irradiation des cellules cancéreuses que du stroma mammaire ont fait augmenter l'agressivité des cellules cancéreuses du sein, confirmant la pertinence d'investiguer et de mieux comprendre les mécanismes de régulation de la tumeur avec son microenvironnement. En lien avec le premier objectif, l'inflammation a été identifiée comme joueur clé dans le phénomène d'invasion radio-induite. La CQ, entre autres par son effet anti-inflammatoire, a permis de prévenir cet effet néfaste des radiations.

Le deuxième objectif de cette thèse a aussi été atteint par l'identification de la MT1-MMP comme biomarqueur potentiel de prédiction des effets de la radiation. En détectant l'expression et la localisation de la MT1-MMP par IHC sur des biopsies de TNBC, il serait possible d'identifier les femmes les plus susceptibles de présenter des risques d'invasion radio-induite. Cela permettrait d’anticiper la radiosensibilité de la tumeur et des tissus sains ainsi que d'optimiser les régimes de RT au sein de ce sous-groupe de patientes.

Bien que les objectifs expérimentaux de cette étude aient été atteints, plusieurs critiques peuvent être énoncées en lien avec mes travaux. Premièrement, cette étude n'est pas directement transférable à l'humain pour plusieurs raisons. La variation interespèce en est la première cause. En effet, cette étude utilise les cellules cancéreuses D2A1 provenant d'un carcinome mammaire de souris et non humain. L'utilisation de cellules humaines pourrait sembler plus appropriée à première vue, mais l'inflammation s'est avérée primordiale dans le phénomène d'invasion radio-induite. L'utilisation de xénogreffes implantées chez la souris immunodéficiente aurait certes été plus représentative d'un cancer humain, mais n'aurait pas reflété fidèlement la réaction inflammatoire post-irradiation en raison du système immunitaire déficient. L'utilisation de souris immunodéficientes humanisées possédant des cellules immunitaires humaines aurait été une alternative très intéressante qui permettrait l'utilisation de cellules cancéreuses humaines dans un contexte inflammatoire représentatif. Finalement, il ne faut pas négliger le fait qu'un modèle animal où l'on implante directement les cellules cancéreuses dans les glandes mammaires ne représente pas la même relation tumeur-stroma qu'une tumeur spontanée.

La faiblesse principale de cette étude est sans aucun doute qu'une seule lignée

cellulaire triple négative a été testée chez l'animal. Bien que nous ayons vérifié le potentiel d'invasion radio-induite de d'autres lignées cancéreuses du sein *in vitro*, il aurait été pertinent d'observer cet effet au moins avec une autre lignée triple négative chez la souris. Il aurait bien sûr été encore mieux de valider que cet effet néfaste des radiations est particulier aux TNBC en utilisant aussi des lignées moins agressives comme des cellules cancéreuses estrogènes-positives par exemple.

Outre le modèle animal, le protocole d'irradiation, n'étant pas le même qu'utilisé en clinique, représente aussi une limitation de l'étude. L'irradiation répétée d'une souris avec autant de fractions que chez l'humain est impossible étant donné que chaque fraction de la dose nécessite l'anesthésie de l'animal. L'isoflurane ou la kétamine administré à chaque jour pendant plusieurs semaines serait potentiellement létal pour l'animal. Bien que les doses ne soient pas exactement les mêmes qu'en clinique, celles-ci ont été calculées à l'aide des physiciens médicaux et des radiooncologues du CHUS pour obtenir une dose biologique comparable à la dose utilisée chez l'humain.

Du côté technique, le décompte de métastases pulmonaires pourrait avoir été sous-estimé. Étant donné que les poumons sont étalés à plat pour la prise des photos en fluorescence, il est possible que certaines métastases se retrouvent dans le même champ de vue, donc comptées comme une seule, sous-estimant ainsi le nombre total. L'utilisation d'une modalité d'imagerie en 3D serait certainement très intéressante pour la poursuite de l'étude. En lien, un groupe de recherche du Centre d'imagerie moléculaire de Sherbrooke est actuellement en train d'optimiser l'imageur optique pour petit animal utilisé dans cette étude. Une nouvelle caméra à dispositif de transfert de charge sera installée sur l'appareil et des algorithmes de reconstruction d'images tomographiques 3D sont actuellement en développement. En ce qui concerne les fluorophores transfectés dans les cellules D2A1 utilisés dans cette étude, la protéine Amazi Green émet de la fluorescence à une longueur d'onde d'environ 550 nm et le tissu pulmonaire émet de l'autofluorescence à cette même longueur d'onde. Il est donc possible que certaines petites métastases aient été confondues dans ce signal non-spécifique. Un meilleur signal aurait été obtenu avec un fluorophore émettant de la lumière entre 650 et 800 nm, soit des longueurs d'ondes pour lesquelles l'autofluorescence des tissus biologiques est considérablement moindre, voire négligeable. Aussi, le décompte de cellules tumorales circulantes s'est effectué par microscopie en

fluorescence. Bien que cette technique permette d'utiliser une très faible quantité de sang, le décompte aurait été plus précis en utilisant la technique de cytométrie en flux. Cette technique était d'ailleurs notre premier choix, mais le volume sanguin nécessaire pour une quantification optimale aurait exigé le sacrifice de l'animal.

En ce qui concerne les courbes de croissances tumorales, la forme très irrégulière des tumeurs peut s'avérer être un facteur d'erreur. Avec sa forme très allongée, la glande mammaire de souris induit souvent une tumeur également de forme allongée. La formule classique de la demi-sphère pour le calcul du volume tumoral devrait donc être révisée pour ce modèle animal. Aussi, il ne faut pas négliger les erreurs de manipulations liées à l'expérimentateur. La précision et l'endroit exact d'injection des cellules cancéreuses peut entraîner une irrégularité de la forme de la tumeur. En comparaison avec le vernier utilisé come instrument de mesure dans cette étude, une technique d'imagerie comme l'IRM aurait été beaucoup plus précise pour mesurer le volume tumoral et aurait permis par la même occasion de fournir des paramètres sur l'invasion de la tumeur dans le tissu environnant. L'IRM permet aussi de calculer des coefficients de perméabilité vasculaire. En lien, cette étude ne parle que brièvement de l'effet des radiations sur les cellules endothéliales. Bien qu'aucun dommage vasculaire n'ait été remarqué sur les coupes histologiques, un épaississement de la paroi des vaisseaux sanguins a tout de même été observé (Figure 2). Une augmentation de la perméabilité des vaisseaux peut faciliter l'entrée de cellules cancéreuses dans la circulation sanguine ou encore attirer des cellules immunitaires qui influenceront à leur tour leur migration (Barker *et al.*, 2015). Bien que l'IRM aurait été beaucoup plus précise comme modalité d'analyse, celle-ci à été exclue car la mise au point s'est avérée très complexe au sein de la glande mammaire de souris. En effet, le tissu graisseux de la glande mammaire induit un signal très intense, occasionnant des artéfacts dans les images. Il serait très intéressant d'optimiser la technique d'IRM pour mesurer l'invasion des cellules cancéreuses du sein dans la glande mammaire et ainsi obtenir ces paramètres supplémentaires.

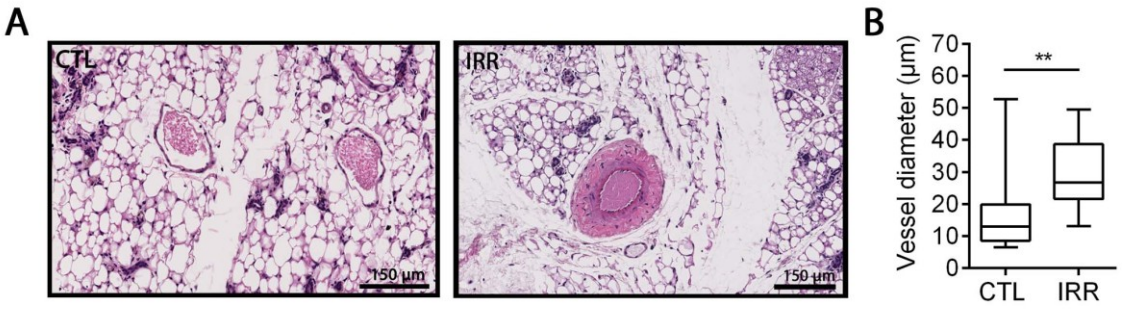


Figure 2. **Épaississement de la paroi vasculaire post-irradiation.** (**A)** Coloration H&E des vaisseaux sanguins 6 h post-irradiation après la dernière fraction de 6 Gy administrée à 24 h d'intervalle pendant 4 jours. (**B**) Quantification de l'épaisseur de la paroi des vaisseaux sanguins en utilisation le logiciel *Nanozoomer Digital Pathology*. La paroi vasculaire est significativement plus épaisse dans les glandes mammaires irradiées (P = 0.0092) en comparaison des glandes mammaires contrôles (n = 3 souris/condition). CTL: contrôle; IRR: irradié.

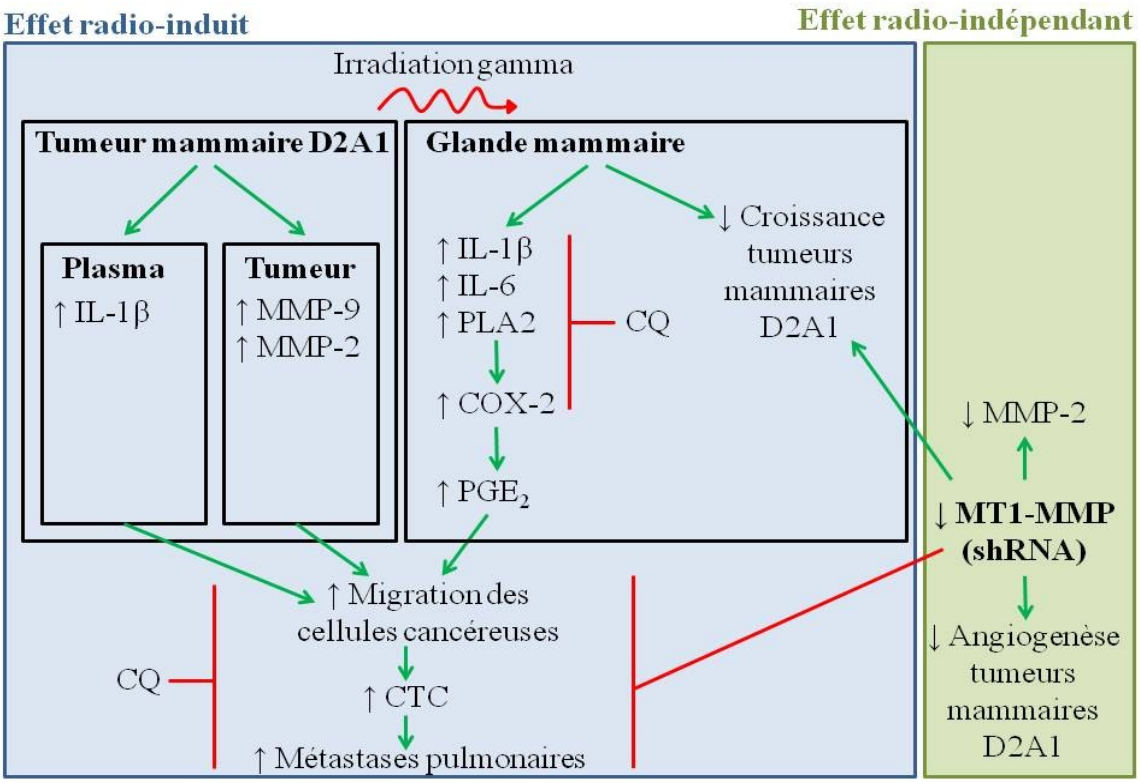
3. CONCLUSION ET PERSPECTIVES

En perspectives de cette étude, une étude pilote est actuellement entamée par notre groupe de recherche. Celle-ci consiste à analyser des biopsies de TNBC en plus d'un bilan plasmatique avant, pendant et après le traitement de RT. Comme le suggère cette étude, l'expression et la localisation de la MT1-MMP sera quantifiée. Conjointement, l'IL-1β plasmatique sera quantifiée mais cette cytokine est normalement induite par le traitement de RT. Nous voulons donc un biomarqueur de prédiction afin d'anticiper l'efficacité du traitement en premier lieu, puis ensuite, la quantification de l'IL-1β serait appropriée pour un suivi plus efficace. En lien avec le rôle de l'IL-1β dans l'invasion radio-induite préalablement mis en évidence dans le premier objectif de cette thèse, il serait pertinent de quantifier l'expression son récepteur au sein des même biopsies TNBC. L'expression du récepteur chez les cellules cancéreuses serait aussi un bon indice de prédiction de la susceptibilité de la tumeur au phénomène d'invasion radio-induite. L'utilisation de plusieurs biomarqueurs des effets de la RT (MT1-MMP, IL-1β et récepteur de l' IL-1β) simultanément augmenterait certainement la fiabilité de la prédiction.

Du côté fondamental, certains mécanismes d'action étudiés dans cette thèse

nécessitent des recherches plus approfondies. Par exemple, il faudrait identifier le processus responsable du passage de la MT1-MMP du cytoplasme vers le noyau. L'endocytose a été proposée par le groupe de Ip *et al.* mais celle-ci reste à confirmer dans cette présente étude. Le mécanisme précis par lequel la CQ inhibe la migration et l'invasion radio-induite reste aussi à confirmer. Afin de savoir si cet effet est aussi MT1-MMP-dépendant, l'expression ainsi que la localisation de la MT1-MMP pourraient être vérifiées par IHC sur des coupes de tumeurs D2A1 traitées à la CQ. Une perte de signal au niveau des noyaux des cellules cancéreuses chez les souris irradiées puis traitées à la CQ serait un bon indicateur que le mécanisme d'inhibition de l'invasion radio-induite de la CQ est MT1-MMP-dépendant.

En conclusion, dans l'optique d'augmenter l'efficacité de la RT administrée aux patientes atteintes de TNBC, l'identification de biomarqueurs de prédiction des effets de l'irradiation est essentiel. Les résultats de cette thèse confirment le potentiel de la MT1-MMP comme biomarqueur de prédiction de l'invasion radio-induite. Les principaux résultats sont résumés à la Figure 3. Ainsi, un traitement anti-inflammatoires (IL-1β, IL-6 et/ou COX-2) pourrait être administré en même temps que la RT dans le but d'optimisation celle-ci. S'il existe une corrélation entre l'expression, la localisation de la MT1-MMP et les échecs de traitement suite à la RT dans les biopsies de patientes TNBC, un biomarqueur des effets de la RT serait identifié pour la toute première fois. Cela représenterait des avancées notables dans les domaines de la radiothérapie ainsi que du cancer du sein triple négatif sachant que peu de traitements sont actuellement disponibles pour ce dernier.



COX-2, cyclooxygénase-2; CQ, chloroquine; CTC, cellule tumorale circulante; IL, interleukine; MMP, métalloprotéinase de matrice; MT1-MMP, métalloprotéinase membranaire de type 1; PGE2, prostaglandine E2; PLA2, phospholipase A2; shRNA, petit ARN en forme d'épingle.

Figure 3.Schéma résumé des principaux résultats de cette étude.

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ANNEXES

Annexe A. Liste des communications

17,18 et 21 mars 2016

Gina Bouchard*, Cheminement vers les études supérieures en sciences de la santé, Polyvalente Jean-Gauthier, Alma (conférencière invitée)

7 mars 2016

Gina Bouchard*, Rachel Bujold, Caroline Saucier, Yves Bérubé-Lauzière and Benoit Paquette Radiation-induced migration and invasion of TNBC cells, laboratoire Plevritis, Université Stanford, CA, USA (conférencière invitée)

19 - 22 septembre 2015

Gina Bouchard*, Rachel Bujold, Caroline Saucier, and Benoit Paquette, Should triple negative breast tumors be treated with radiation or not?, Radiation Research Society, 61th Annual Meeting, Weston, FL, USA (affiche)

20 - 24 septembre 2014

Gina Bouchard*, Rachel Bujold, Yves Bérubé-Lauzière, Caroline Saucier, and Benoit Paquette, MT1-MMP: a new biomarker of ionizing radiation in triple negative breast cancer patients, Radiation Research Society, 60th Annual Meeting, Las Vegas, USA (affiche)

3 avril 2014

Gina Bouchard*, Rachel Bujold, Yves Bérubé-Lauzière, Caroline Saucier, and Benoit Paquette, MT1-MMP: un nouveau biomarqueur de prédiction des effets de la radiothérapie?, Symposium de recherche sur le cancer, Sherbrooke, Canada (oral)

6 - 10 octobre 2013

Gina Bouchard*, Rachel Bujold, Caroline Saucier and Benoit Paquette, Prévention de la migration radio-induite des cellules cancéreuses du sein, 11ème conférence internationale de radiobiologie fondamentale et appliquée. Montpellier, France (oral)

18 - 21 septembre 2013

Benoit Paquette*, Gina Bouchard and Caroline Saucier. Pre-irradiation of the mammary gland in mice increases the invasiveness of breast cancer cells and lung metastases, The Canadian Association of Radiation oncology, 2013 Annual Scientific Meeting, Montréal, QC, Canada (oral)

28 septembre 2013

Benoit Paquette*, Gina Bouchard, Hélène Therriault, Rachel Bujold et Caroline Saucier. New avenues of research to improve the efficacy of radiotherapy

Potential role of radiation in breast cancer progression. Shandong Radiation Oncology Conference, Jinian, Chine (conference invitée).

3 - 6 novembre 2013

Benoit Paquette*, Gina Bouchard, Hélène Therriault, Rachel Bujold, Caroline Saucier. Pre-irradiation of mouse mammary gland stimulates the migration of triple negative breast cancer cells and development of lung metastases. The Canadian Cancer Research Conference. Toronto, Ontario, Canada (oral)

30 septembre - 3 octobre 2012

Gina Bouchard*, Rachel Bujold, Caroline Saucier and Benoit Paquette. Pre-irradiation of Balb/c mice mammary gland increases the invasiveness of breast cancer cells and the number of lung metastases, Radiation Research Society, 58th Annual Meeting, Puerto Rico, USA (affiche)

12 - 15 septembre 2012

Benoit Paquette*, Gina Bouchard and Caroline Saucier. Pre-irradiation of the mammary gland in mice increases the invasiveness of breast cancer cells and lung metastases. The Canadian Association of Radiation oncology, 2012 Annual Scientific Meeting, Ottawa, ON, Canada (oral)

11 - 16 septembre 2011

Gina Bouchard*, Rachel Bujold, Caroline Saucier and Benoit Paquette. Rôle de la MT1-MMP et du TGF-β1 dans l'augmentation radio-induite de la migration et de l'invasion des cellules cancéreuses du sein. 10ème conférence internationale de radiobiologie fondamentale et appliquée. Anglet, France (oral)

9 novembre 2011

Gina Bouchard*, Rachel Bujold, Caroline Saucier and Benoit Paquette, Prévention de l'invasion radio-induite des cellules cancéreuses par la chloroquine, Journée Phare , Orford, Canada (oral)

6-8 octobre 2011

Benoit Paquette*, Gina Bouchard, Rosalie Lemay, Rachel Bujold, Hélène Therriault, Luc Tremblay and Martin Lepage. Inflammation induced by radiation enhances breast cancer cell invasion, 16th World Congress on Advances in Oncology and 14th International Symposium on Molecular Medicine. Rhodes, Greece (oral)

*présentateur

Annexe B. Liste des prix et bourses

- Bourse d'étude universitaire, *Fondation Jacques Gagnon* 2016
- Bourse d'étude doctorale, *Fonds de la recherche du Québec-Santé* 2013-2016
- Bourse de voyage, *Instituts de recherche en santé du Canada* 2015
- Scholars in Training Travel Award, *Radiation Research Society* 2015
- Scholars in Training Travel Award, *Radiation Research Society* 2014
- Meilleure publication de l'année, Institut Cancer, *Instituts de recherche en santé du Canada* 2014
- Meilleure présentation orale (3e place*),* Symposium de la recherche sur le cancer, *Université de Sherbrooke* 2014
- Mention d'honneur du doyen, Programme de sciences des radiation et imagerie biomédicale, *Université de Sherbrooke* 2013
- Scholars in Training Travel Award, *Radiation Research Society* 2012
- Meilleure présentation orale, Jeune scientifique 2011, Prix-Joseph Maisin, *Société de radiobiologie de la langue française* 10ème CIRFA, Anglet, France 2011
- Meilleure présentation par affiche catégorie physiologie/pharmacologie, journée scientifique du premier cycle, *Université de Sherbrooke* 2011
- Bourse d'étude, *Centre de recherche en radiothérapie (CR^2),* Université de Sherbrooke 2011
- Bourse d'étude, *Centre de recherche en radiothérapie (CR^2),* Université de Sherbrooke 2010
- Bouse d'étude, *Coopérative financière Desjardins* 2006